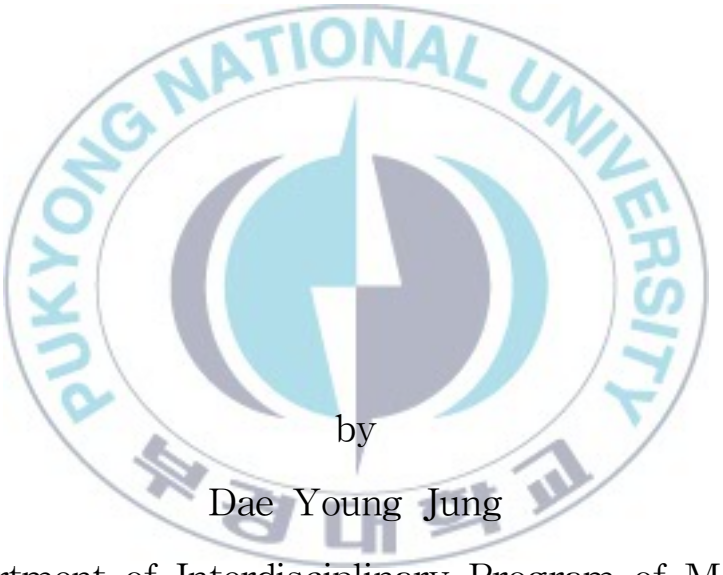
Thesis for the Degree of Doctor of Philosophy

Development of the process for mass production of various molecular weight pullulan by *Aureobasidium pullulans* HP-2001



by

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Department of Interdisciplinary Program of Marine Biotechnology

The Graduate School

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February 2010

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Aureobasidium pullulans HP-2001에 의해 생산되는 다양한 분자량의 풀루란 대량 생산 공정에 관한 연구

Advisor: Prof. Sung-Koo Kim

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A dissertation

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국문초록

Aureobasidium pullulans HP-2001에 의해 생산되는 다양한 분자량의 풀루란 대량 생산 공정에 관한 연구

정 대 영

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풀루란 대량생산의 특성을 확인하기 위해 7 L, 100 L, 5 ton, 6 ton air-lift 타입의 배양기를 이용하여 최적의 대량생산 공정을 확립하고 생성되는 풀루란의 배양중에 분자량을 조절하는 연구를 진행 하였다.

sucrose를 탄소원으로한 최적화된 배지조성으로 7 L 발효조의 교반속도를 600 rpm으로 배양했을 경우, 72시간에 이르러 최고 60.80 g/L의 pullulan을 얻을 수 있었다. 또한 Scale up을 통한 6 ton air-lift 타입의 발효기에서는 48.80 g/L의 pullulan을 얻었다.

최근에는 저분자화 또는 고분자화하여 생물학적 기능을 이용한 새로운 형태의 다양한 기능성 소재들이 개발되어 제품화 되고 있으며, 각 분자량에 따라 이용도가 달라진다. 따라서 본 연구에서

초기 pH, 배양 중 pH 조절, $CaCO_3$와 Ascorbic acid 첨가에 의한 배양중 분자량 조절, 가수분해 및 기질에 의한 분자량 조절 기술에 관한 연구를 진행 하였다. 풀루란의 분자량의 조절에 있어서 가장 핵심적인 요소는 배양중에 pH 변화를 어떻게 조절하는가와 깊은 관련을 가지고 있다. 이에 배양 초기 pH 조절 연구 결과 pH 5-6에서 생산성이 가장 높게 나왔으며 분자량은 800-1,000 KDa 풀루란이 생산되는 결과를 보여주었다. 여기서 주목할 것은 초기 pH가 낮을수록 고분자의 풀루란이 생산되는 것을 확인할 수 있었고 반면에 생산성은 감소하는 경향을 나타내었다. 따라서 초기 pH를 6.0으로 고정하고. 배양 중 pH를 변화시키는 방법으로 인위적으로 $CaCO_3$를 첨가하여 조절하였다. 그 결과 1,000 KDa 정도의 고분자 풀루란을 생산할 경우 질소원으로 ISP를 2.5g/L 첨가하거나 $CaCO_3$ 0.1g/l을, 500kDa의 풀루란을 생산할 경우 $CaCO_3$ 0.5g/l을, 300kDa 내외의 풀루란을 생산할 경우 $CaCO_3$ 1.0g/l 혹은 Ascorbic acid를 혼용하여 사용하는 것이 가장 효과적이었다. 또한 배양 중에 발생하는 색소 침착 방지를 위해 ascorbic acid를 첨가하였으며 1 g/L 가 가장 효과적임을 확인하였다.

발효 공정 후 분리 정제 과정에서는 균체 및 색소침착이 제거된 순수한 풀루란 생산을 목적으로 한다. 이에 여과보조제로 규조토를 첨가한 배양액을 필터프레스를 이용하여 균체를 제거하고, 활성탄 처리를 통한 탈색, 탈취공정을 진행하였다.

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Development of the process for mass production of various molecular weight pullulan by *Aureobasidium pullulans*

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Abstract

Aureobasidium pullulans HP-2001 used in this study was the UV-induced mutant of *A. pullulans* ATCC 42023. For the production of pullulan by *Aureobasidium pullulans* HP-2001, the culture characteristics of *A. pullulans* and mass production of pullulan in batch fermentations using 7 liter, 100 liter, 5 ton fermenters and 6 ton air-lift fermenter through the optimization of the fermentation process were investigated. The optimal condition of pullulan fermentation by continuous culture was evaluated.

The production of pullulan by *Aureobasidium pullulans* HP-2001 was studied using optimized conditions with

sucrose medium. The production of pullulan with the highest conversion rate was 39.95 g/l when concentrations of sucrose and yeast extract were 10% and 0.25% (w/v), respectively The average molecular weight of pullulan ranged from 6.50×10^3 to 5.87×10^6 depending on the culture conditions. The cells from the culture with an initial pH 6.0 had the highest ability to produce pullulan among the cultures with different initial pH values. Agitation speed affected the dissolved oxygen in the medium of a fermenter. High agitation speed increased the production of pullulan. Maximal production of pullulan with the optimized condition by *A. pullulans* HP-2001 in a 7 liter fermenter was 60.80 g/l for 72 hr culture with an agitation speed of 600 rpm. In a 6 ton air lift fermenter, the aeration rate was optimized to 1.0 vvm at the initial pH of 6.0. Dissolved oxygen was decreased during the fermentation and decreased to 60% after 12 hr of fermentation. Maximal production of pullulan by *A. pullulans* HP-2001 in a 6 ton fermenter was 48.80 g/l.

Continuous culture of *A. pullulans* HP-2001 for the production of pullulan was studied in a 7 liter fermenter. The optimal conditions of continuous culture for pullulan fermentation were determined by the effects of composition,

dilution rate and sucrose concentration of feed solution. The optimal dilution rate for the production of pullulan was 0.015 h^{-1} and higher dilution rate caused a wash out of cells. The average molecular weights of pullulans produced by a continuous fermentation ranged from 2.98×10^5 to 5.40×10^5.

The objectives for this study was to evaluate the optimal condition for the production of pullulan with specific molecular weight in a fermentation. The portion of high molecular weight of pullulan increased at the early phase of the culture. The pullulan molecular weight decreased at stationary phase due to the enviromental factors. The pH control of culture broth was one of the most critical environmental parameters affecting molecular weight. The pullulan production increased by the increase of initial pH from 3 to 5. However, a high proportion of high molecular weight pullulan (M.W 1.64 x 10^6) was produced at a pH of 3. In order to control the pH during the fermentation 20g/l to 0 of $CaCO_3$ was added. The addition of less than 1 g/l of $CaCO_3$ increased the portion of high molecular weight of pullulan production . On the other hand, Further $CaCO_3$ addition decreased the molecular weight of pullulan .

The high molecular weight of the pullulan fermentation was influenced not only $CaCO_3$, ascorbic acid but also substrate. Overall the molecular weights of pullulan produced with isolated soybean protein (ISP) was 4 times higher than those of pullulans produced with yeast extract as a nitrogen source.

Melanin-like pigment was produced with pullulan production, which contaminates the pullulan. A decolorization process using ascorbic acid during fermentation was tried to remove the pigment. Ascorbic acid plays the role of an effective antioxidant in biological systems. The ascorbic acids concentrations ranged from 0.0 to 3 g/l were added to eliminate the colorization of fermentation broth. The optimal concentration of ascorbic acid was 1 g/l.

A microbial fermentation and recovery process were developed for large-scale production. Downstream processing is required to obtain pure pullulan from the fermentation broth and it comprises of cell separation from culture broth after cultivation and the removal of melanin pigments produced during fermentation.

The optimal process for the concentration process of pullulan from filtrates after removing of cells by a filter press

was carried out by a ceramic membrane with a molecular cut off size of 50 kDa to remove materials and concentrate pullulan. As a Final process, freeze drying was carried out using the filtrate.

I. INTRODUCTION

1. Production of Pullulan

Pullulan which is a linear α-D-glucan with 'maltosyl units' i.e. α-(1→4)Glup-α-(1→4)Glup-α-(1→6)Glup, as a regularly repeating structural unit, produced extracellularly by *Aureobasidium pullulans*. Thus, the polysaccharide is viewed as a succession of α-(1→6)-linked (1→4)-α-D-triglucosides i.e. maltotriose (G3). Due to its strictly linear structure, pullulan is very valuable in basic research as well as a well-defined model substance [1](Table 1). Pullulan can form thin films which are transparent, colourless, tasteless, odorless, tenacious, resistant to oil and grease and unaffected by small thermal variations. Besides, the films are also impermeable to oxygen, non-toxic, biodegradable and edible [2]. It is insoluble in many solvents including methanol, ethanol and acetone, but soluble in water to form a transparent, colourless, viscous adhesive solution [3]. Nowadays pullulan has been gaining attention as an excellent material for food, pharmaceutical and biomedical applications [4]. Fig. 1 represents the fine structure of pullulan which is

Table 1. The structure of some bacterial polysaccharides

Species	Polysaccharide	Basic structure
Leuconostoc mesenteroides	Dextran	(1-6 glucose)n
Acetobacter xylinum	Cellulose	(1-4 glucose)n
Alcaligenes faecalis var. myxogenes	Curdlan	(1-3 glucose)n
Streptococcus salivarius	Levan	(2-6 fructose)n
Azotobacter vinelandii	Alginate	1-4 linked ManA and GulA
Streptococci (Haemolytic Group A)	Hyaluronan	(-3GluNAc1-4GlcA1-)n
Xanthomonas campestris	Xanthan	(-4Glc1-4Glc1-4Glc1-)n

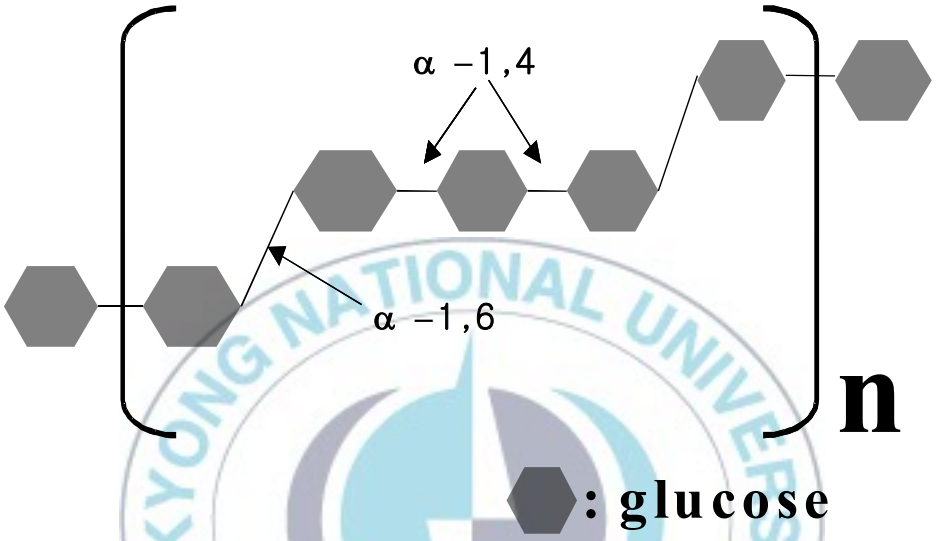


Fig. 1. Chemical structure of pullulan

generally accepted. This polysaccharide was characterized as the homopolymer of glucose and termed as pullulan. Investigators calculated the degree of polymerization from 100 to 5,000 and the structure and molecular weight of pullulan seemed to be prone to variation by cultivation conditions such as carbon sources, the pH of the culture broth and the concentration of inorganic nutrients, etc.

The purified pullulan product is obtained in a tasteless, odorless, white and fine powder by subjecting the culture broth to a sufficient period of cultivation, cell separation and precipitation with solvent (Fig. 2). Typical strains that produce pullulan at a relatively high yield include *Pullularia pullulans*, *Pullularia fusca*, *Pullularia fermentans* and *Dematium pullulans*.

However, there are several undesirable features associated with the fermentation of *A. pullulans* [5,6]. These include the formation of a melanin-like pigment, the inhibitory effects caused by high sugar concentration, the decline in the molecular weight of pullulan as fermentation progresses and the high cost associated with pullulan precipitation and recovery. In polysaccharide fermentations, the utilization of the high concentration of sugars without the decrease of the production

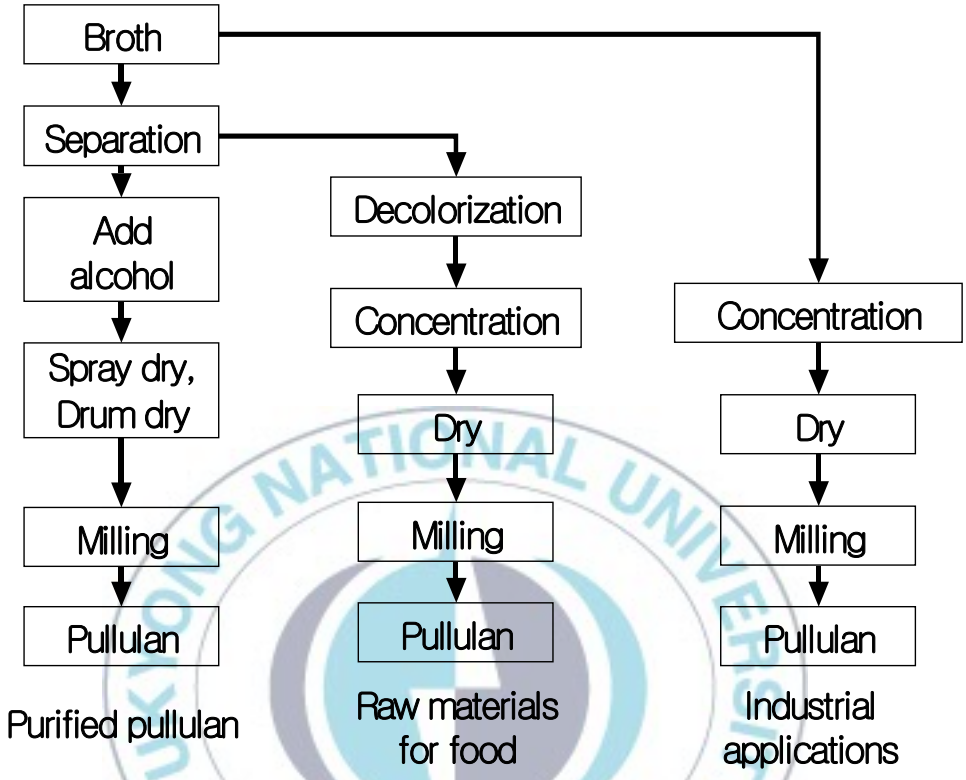


Fig. 2. Production and purification of pullulan

rate of polysaccharide has some advantages for saving the solvent used for the recovery of polysaccharide.

Pullulan can be used as a film, food coating and packing agents, food ingredients, adhesives and fabric. The controlling of pullulan molecular weight is the most important technique during the process of pullulan production. Pullulan produces a high viscosity solution at a relatively low concentration and can be used for oxygenimpermeable films, thickening or extending agents or adhesives [7]. Despite these advantages, the applications were actually limited due to the low production yield and the low molecular weight of the pullulan.

2. Uses and Application of Pullulan

Current industrial interests concerned with polysaccharide have increased, as it has become a very interesting research item for industrial application because polysaccharide is produced by microorganisms that has various kinds of type and functional characteristics. Pullulan dissolves easily in cold water to form a neutral, non-ionic, aqueous solution. Thermal decomposition of pullulan starts at close to 250℃, similarly to the case of starch. Because pullulan is non-toxic to human, it can be used as a safe food ingredient or additive [8].

Microbial biopolymers are known to possess useful physical properties, even then currently only a small number of biopolymers are produced commercially on large scale. A few fungal EPSs have been reported so far that possess appealing industrial applications. Pullulan, a water soluble biopolymer from A. pullulans is one of such fungal EPSs. Numerous applications of pullulan in food and pharmaceuticals manufacturing have been reviewed [2](Table 2).

Table 2. Uses and applications of pullulan

Properties	Foods	Effects
Adhesive strength	Rice cake Various foods	●Strong adhesive property, Improvement of taste ●Adhesiveness, Binding capacity ●Prevention of oxidation, Gloss
Coating	Dried fish and shellfish Dried fishes	●Gloss, Prevention of oxidation
Viscosity	Sauce Hard-boiled food Cookies Salted vegetables Sea foods	●Improvement of adhesion, Gloss ●Gloss maintained, Prevention of spilling Moldability ●Prevention of fragility ●Gloss, Salt tolerance, Enzyme tolerance ● Gloss, Prevention of discoloration
Water holding	Breads, Sponge cake Gum Frozen foods Boiled fish pasta Noodle	●Moisturizing effect, Improvement of taste, Stabilization of bubble ●Prevention of degradation, Improvement of quality ●Improvement of quality, Prevention of drip ●Improvement of taste, Prevention of aging Prevention of aging ●Improvement of water-holding property, ●improvement of quality

2-1. Food Industry

Pullulan provides few calories and is treated as dietary fiber in rats and humans [9,10] This is because of its resistance to mammalian amylases. Studies indicate that dietary pullulan functions as prebiotic, promoting the growth of beneficial bifidobacteria [10,11,12]. Pullulan may be incorporated in solid as well as liquid food to replace starch; imparting the characteristics to food normally derived from starch as consistency, dispersibility, moisture retention, etc. Pullulan improves the shelf life of the food as it is not a readily assimilable carbon source for bacteria, molds and fungi responsible for spoilage of food. Pullulan is also superior to starch in water retention thus retarding the spoilage of food by drying out [13,14,15,16]. Pullulan solutions resemble gum arabic having relatively low viscosity [17]. Pullulan can be used as low-viscosity filler in beverages and sauces. It can also be used to stabilize the quality and texture of mayonnaise. The viscosity of pullulan is not affected by heating, changes in pH and most metal ions, including sodium chloride. Adhesive properties are also exhibited by pullulan and its derivatives [15]. Pullulan can be used as a binder and stabilizer in food pastes;

it can also be used to adhere nuts to cookies. Pullulan can be employed as a binder for tobacco [18], seed coatings and plant fertilizers [19].

Pullulan films are clear and highly oxygen-impermeable with excellent mechanical properties. The oxygen resistance of pullulan films is suitable for the protection of readily oxidized fats and vitamins in food. Pullulan films can be employed as coating or packaging material of dried foods, including nuts, noodles, confectionaries, vegetables and meats [20]. Pullulan can be used directly to foods as a protective glaze[21,22]. Pullulan is referred as slowly digested carbohydrate as human enzymes gradually convert pullulan to glucose that results in gradual rise in blood glucose level in humans. Pullulan may be incorporated into dietetic snack foods designed for diabetics. Pullulan is also beneficial to patients who have impaired glucose tolerance [23].

2-2. Pharmaceutical

Pullulan and its derivatives can be used as a denture adhesive. Adhesives or pastes are prepared by dissolving or dispersing uniformly pullulan ester and/or pullulan ether in

water or in a mixture of water and acetone. Adhesives and pastes containing pullulan as the main component have higher water solubility and lower moisture resistance [24]. Sugar-coated pharmaceutical compositions such as tablets, pills, granules contain pullulan in the sugar layer for the purpose of preventing brownish color change of the composition with lapse of time. The solid sugar-coated preparation exhibits an enhanced impact strength and shelf life. Pullulan can also be used for pharmaceutical coatings, including sustained-release formulations. Novel preparations such as tablets, pills, granules or the like, which contain pullulan in the sugar layer serve the purpose of preventing brownish color change of the composition [25,26,27]Oral care products have been commercialized based on pullulan films. The colorless, transparent and edible pullulan film has also attracted a great deal of interest for other uses such as a non-polluting wrapping material [28,29] reported pullulan compositions for the use in pharmaceutical products preferably for predosed formulations like soft and hard capsules. Pullulan derivatives are promising as non-toxic conjugates for vaccines [30,31]. Covalent attachment between the virus and pullulan remarkably enhances the inherent producibility of

immunoglobulin G and immunoglobulin M antibodies and diminishes the immunoglobulin E antibody producibility as well as sufficiently inactivating and detoxifying the virus. Pullulan can provide liposome delivery [32,33]. Sized pullulan fractions having molecular weight 30,000 to 90,000 Da can be used as a blood plasma expander in place of dextran [34,35]. There have been several attempts to develop plasma substitutes on pullulan [3,36] summarized all efforts that have been made so far to understand the pharmacokinetics of intravenously applied pullulan in terms of the molar mass and concentration [37]. has demonstrated the use of pullulan in cosmetics, lotions and shampoos. Pullulan being non-toxic and non-irritant to human body, may be applied to any cosmetics, but is preferably used as an ingredient of cosmetic lotions, cosmetic powders, cosmetics around eyes, facial packs, shampoos, specific hair dressings (set lotions and hair lacquers), and tooth powders. Excellent transparent film-forming ability, moisture absorptivity, water solubility and tackiness are the properties making pullulan suitable for use in cosmetics.

2-3. Miscellaneous Industry

Pullulan is also used as an industrial flocculating agent [38,39]. It can also be used in the production of paper. The invention pertains to novel paper-coating material containing pullulan which is excellent in gloss, printing gloss, adhesive strength and viscosity stability during storage. Pullulan has excellent properties as a paper-coating adhesive. The pullulan paper is high in strength and folding resistance, is tougher than a wood pulp paper. It favors ink receptivity because of its high hydrophilic nature, hence making it suitable for printing and writing [40,41]. It can improve the characteristics of paint [42]. Pullulan and its derivatives also have photographic, lithographic and electronic applications [43,44,45,46,47]. Pullulan and pullulan derivatives are superior to traditionally used gum Arabic solution in the protection of the surface of lithographic printing plate against oxidation and scumming as well as in the ability to enhance the hydrophilic character of metallic surface of a non-image area [48,49] reported the use of cross-linked pullulan beads (analogousto Sephadex^{R}) in gel permeation chromatography. Cross-linked pullulan is water-resistant without loss of its excellent properties such as high degrees of

transparency, toughness and adhesiveness. Pullulan gels have been used for enzyme immobilization. Hydrophilic pullulan gel having a three-dimensionally reticulated structure which is obtained by the reaction between pullulan and a bifunctional compound capable of forming an ether linkage with the hydroxyl group present in glucose unit of pullulan is used as a carrier. Enzymes immobilized with pullulan gel have a high activity and good retention of activity [50].

2-4. Control of Molecular Weight Pullulan

Pullulan application was actually limited due to low molecular weight of the pullulan. Some important factors that control the molecular weight of the pulluan from *A. pullulans* are temperature, the initial pH of the medium, the concentration and the kind of nitogen source and the carbon source [51]. The average molecular weight of pullulan ranges from 1.5 x 10^4 to 1.0 x10^7 depending on the culture condition and strain used [52]

The factors of culture condition that control molecular weight pullulan have not been well studied, except for high molecular weight pullulan. Several factors for the synthesis and secretion mechanism of pullulan and cellular metabolism of *A.*

pullans have been reported [5]. The pH of the culture broth influences not only the production of pullulan, but also the morphology of A. pullulans. The mycelial form and yeast-like form were predominant at pH 2.0-2.5 and pH 6.0-8.0, respectively. There were several reports on the relationship between the production of pullulan and its morphology [53]. However, these reports showed somewhat conflicting results. Therefore, further study on the relationship between the pullulan production and to obtain various molecular weight pullulan.

In this study, the effects of pH on the production of pullulan, and the molecular weight distribution of pullulan during the fermentation process were evaluated to obtain the optimum molecular weight controlled pullulan fermentation condition.

II. MATERIAL AND METHODS

1. Bacterial Strain and Media

Aureobasidium pullulans HP-2001 isolated in this study was the UV-induced mutant of *A. pullulans* ATCC 42023. It showed the increased production of pullulan and did not produce the melanin-like black pigment. It was transferred monthly to the nutrient agar medium [54]. The medium used for cell growth and the production of pullulan contained the following components (g/l): K_2HPO_4, 5.0; NaCl, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)_2SO_4$, 0.6 and yeast extract (Difco Lab., Detroit. USA), 2.5 [54]. The medium was adjusted to pH 7.0 with 1 N HCl solution. The carbon source was autoclaved separately for 15 min at 121℃ and added to the medium under aseptic conditions. According to the experimental purpose, the kinds and concentrations of carbon sources and nitrogen sources were varied but the concentrations of salts were not changed(Table 3).

Table 3. Composition of seed and main culture medium

	Seed culture	Main culture
Carbon source	2% glucose	10% sucrose
Nitrogen source	0.25% yeast extract	0.25% yeast extract
Salts	0.5% K_2HPO_4 0.1% NaCl 0.02% $MgSO_4 \cdot 7H_2O$ 0.06% $(NH_4)_2SO_4$	0.5% K_2HPO_4 0.1% NaCl 0.02% $MgSO_4 \cdot 7H_2O$ 0.06% $(NH_4)_2SO_4$
Initial pH	7.0	6.0 - 7.0
Working condition	30℃, 200 rpm, 24 hr culture	30℃, 200 rpm, 72-120 hr culture

2. Analytical Methods

2-1. Production of Pullulan

Starter cultures were prepared by transferring cells from agar slants to 50 ml mediums containing 2% (w/v) glucose in 250 ml Erlenmeyer flasks. The resulting cultures were incubated for 2 days at 30℃ and 200 rpm. Each starter culture was used as an inoculum for 100 ml of medium in 500 ml Erlenmeyer flasks. Samples were periodically withdrawn from the cultures to examine cell growth and the production of pullulan.

The culture broth was centrifuged at 15,000 × g for 15 min to remove cells. Supernatant was mixed with 2 volumes of isopropyl alcohol and incubated at 4℃ for 24 hr to precipitate the crude product which was separated by centrifugation at 15,000 × g for 20 min. The precipitated material was repeatedly washed with acetone and ether, dissolved in deionized water (DW) and dialyzed against DW by using dialysis tubing with a molecular weight cut off of from 14,000 to 12,000. After dialysis for 2 to 3 days with four or five changes of DW, the solution

was lyophilized.

To determine biomass, the cells were washed with distilled water and dry cell weight (DCW) measured by directly weighing the biomass after drying to a constant weight at a 100℃ to 105℃. The concentration of pullulan was determined colorimetrically by the phenol-sulfuric acid method [55]. A standard curve for quantitation of pullulan was prepared from the authentic pullulan (Sigma, St. Louis, USA).

2-2. Determination of Sugars

The phenol-sulfuric acid method [55] was used for the measuring the total residual sugar. Reducing sugars contents in the culture broth were determined by the 3,5-dinitrosalicylic acids (DNS) method [56]. DNS reagent was prepared by first dissolving 7.46 g of 3,5-DNS and 13.98 of NaOH pellets in 1 liter of deionized water. Then 216.1 g of Rochelle Salt (sodium potassium tartrate tetrahydrate), 5.38 ml of saturated phenol and 5.85 g of sodium metabisulfite were added, and the reagent was aged for 2 weeks. DNS reagent was added to the same volume of an enzyme-substrate solution, and the preparation was placed in a boiling waster bath for 15 min. After the preparation

cooled to room temperature, the concentration of reducing sugars was determined spectrophotometrically at 550 nm with a spectrophotometer (Unicam Co., Helios Delta, UK).

When sucrose was used as a carbon source, the residual sucrose of the culture broth was determined by Sucrose Assay Kit (Sigma, St. Louis, USA), which is for the quantitative and enzymatic determination of sucrose in foods and other materials [57].

2-3. Composition Analysis by GC and FTIR

Gas chromatography (GC) was used to determine the monomeric composition of sugar repeat units in the pullulan after methanolysis of the oligosaccharide followed by trimethylsilylation (Chaplin, 1982). Gas chromatographic analyses were performed on a Hewlett Packard (HP) gas chromatograph, model 5890 Series II, equipped with a flame-ionization detector, and an HP model 7673 injector. The column was 30 m × 0.32 mm I.D. fused silica with 0.25 mm cross-linked 5% phenylmethyl silicone liquid phase (Supelco, Bellefonte, USA). Dry oxygen-free nitrogen (flow rate of 2.9 ml/min) was used as the carrier gas at 10 psi head pressure using

a temperature program (from 140℃ for 2 min to 260℃ at an increase rate of 8℃ per min). The injector was purged for 0.8 min after injection. Standard curves were constructed of the GC peak area ratio relative to the internal standard of *m*-inositol for various concentrations of pure sugar standards.

Fourier transform infrared (FTIR) spectra were recorded with a Perkin-Elmer 1720 spectrometer (16 scans; resolution, 2 cm^{-1}) over KBr pellets. Pullulan sample (2 mg) was well blended manually with 100 mg of KBr powder. This mixture was then desiccated overnight at 50℃ under reduced pressure prior to FTIR measurement.

2-4. Measurement of Viscosity

Brookfield viscometer, model LVDV-1+, equipped with s18 spindle was used for measuring the viscosity. At 12 hr intervals during the fermentation period, 8 ml of samples were put in a small sample adapter and kept at 25℃. After the deflection had reached a steady value at speed of 6 rpm, the apparent viscosity was calculated.

2-5. Determination of Molecular Weight by Gel Permeation Chromatography (GPC)

The number average molecular weight (Mn) (average molecular weight divided by the number of molecules) and the weight average molecular weight (Mw) (average molecular weight divided by the weight of each polymer chain), as well as the polydispersity (Mw/Mn) (the breadth of the molecular weight distribution) of the pullulan samples, were determined by gel permeation chromatography (Viscotek, USA) equipped with a TSK PW_{XL} column (Viscotek, USA) and a RI detector. Pullulan standards with narrow polydispersity and molecular weights ranging from 5.80×10^3 to 1.60×10^6 were used to construct a calibration curve. Deionized water was used as a mobile phase at a flow rate of 1.0 ml/min. The sample concentration and injection volume were 5.0 mg/ml and 100 μl. All of the sample solutions were filtered through 0.45 μm-pore-size filters (Adbentec MFS, Inc., Japan) before injection.

2-6. Treatment of Pullulans with Pullulanase

Pullulans were assayed for sensitivity to the pullulanase from

Klebsiella pneumonia (Sigma Chemical Co., USA)[59]. Pullulans were suspended at a concentration of 1 mg/ml in 50 mM sodium acetate buffer (pH 5.0). The concentration of the pullulanase was 0.1 U/ml. After mixing, treated samples were incubated for 10 hr at 25℃. Data was reported below as percentages of reducing sugars relative to complete hydrolysis to maltotriose units. Authentic pullulan (Sigma Chemical Co., USA) was used for digestion as a control. Reducing sugar contents were determined by the DNS method [56].

2-7. Removing the Melanin-like Pigments

The supernatant obtained as above was heated to 80℃ for 1 h to deactivate the extracellular pullulanase, filtered through Whatman filter paper no. 2 using a vacuum of 400 mmHg, and then cooled to 25℃. The optical density of this supernatant was measured at 320 nm in a Hitachi Spectrophotometer as an indicator for melanin-like pigmentation. 10 ml of this suspension was precipitated using 20 ml absolute ethanol. The supernatant was decanted, and the residue obtained was dried till constant weight in an oven at 60℃ to give the crude pullulan.

III. RESULTS and DISCUSSION

1. Mass Production of Pullulan from Sucrose Medium by *Aureobasidium pullulans* HP-2001

1-1. Production of Pullulan with Various Concentrations of Sucrose and Glucose as Carbon Source

1-1-1. Method

The medium used for cell growth and production of pullulan contained the following components (g/l) : K_2HPO_4, 5.0; NaCl, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)_2SO_4$, 0.6 and yeast extract (Difco Lab., Detroit. USA), 2.5. The pH of medium was adjusted 6.8 to 7.0 before sterilization. Cultivated at 30℃, 200 rpm in a shaking incubator for 96 hr. The concentration of glucose and sucrose ranged from 0.0 to 20.0% (w/v). The nitrogen source was 0.25% (w/v) yeast extract. As previously investigated, glucose and sucrose were superior to the other carbon sources tested in

regard to its ability to stimulate the production of pullulan.

The medium used for cell growth and production of pullulan contained the following components (g/l) : sucrose, 100; K_2HPO_4, 5.0; NaCl, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)_2SO_4$, 0.6. The pH of medium was adjusted 6.8 to 7.0 before sterilization. Cultivated at 30℃, 200 rpm in a shaking incubator for 96 hr. The concentration of yeast extract ranged from 0.0 to 0.50% (w/v).

The medium used for cell growth and production of pullulan contained the following components (g/l) : sucrose, 100; K_2HPO_4, 5.0; NaCl, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)_2SO_4$, 0.6. Initial pH values ranged from 3.0 to 8.0 in the medium

1-1-2. Results

1-1-2-1 Production of Pullulan with Various Concentrations of Sucrose and Glucose as Carbon Source

The effects of various concentrations of glucose and sucrose as a carbon source on cell growth and production of pullulan by *A. pullulans* HP-2001 were investigated (Fig. 3). The nitrogen source was 0.25% (w/v) yeast extract. As previously investigated, glucose

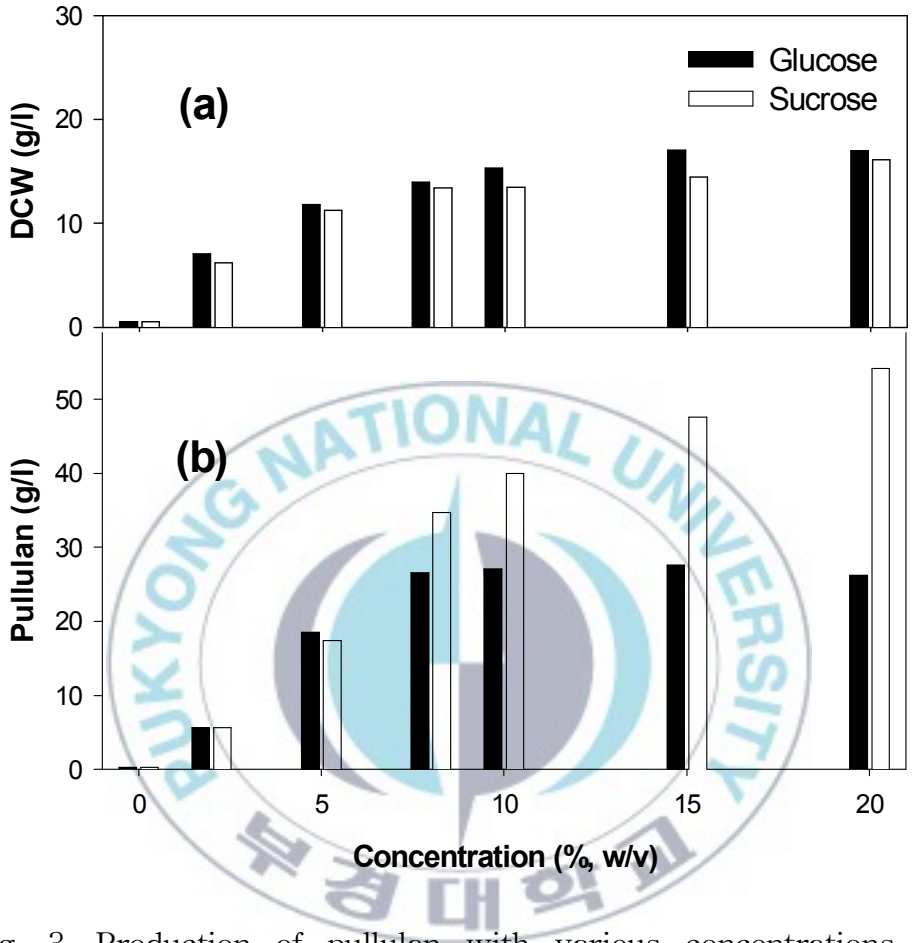


Fig. 3. Production of pullulan with various concentrations of sucrose and glucose as a carbon source in a flask culture. (a) Dry cell weight, (b) Production of pullulan. Cultivated at 30℃, 200 rpm in a shaking incubator for 96 hr.

and sucrose were superior to the other carbon sources tested in regard to its ability to stimulate the production of pullulan. When the carbon source was glucose, production of pullulan increased with increased concentration of glucose up to 10.0% (w/v). Production of pullulan was 27.14 g/l and its conversion rate was 27.1%. When the concentration of glucose exceeded 10% (w/v), the production of pullulan was nearly constant.

Whereas production of pullulan increased with the increased concentration of sucrose as carbon source, catabolite repression was not seen until 20% (w/v) sucrose. Maximal production of pullulan was 54.20 g/l when the concentration of sucrose was 20% (w/v) but its conversion rate was lower than that of 10% (w/v) sucrose. Production of pullulan with 10% (w/v) sucrose as the carbon source was 39.95 g/l when the concentration of yeast extract was 0.25% (w/v) and the ratio of sucrose to yeast extract was 40 : 1. A higher concentration of sucrose resulted in higher production of pullulan but its conversion rate of pullulan was low. In conclusion, the production of pullulan with sucrose was greater than that with glucose under the high initial concentration of carbon sources [57].

1-1-2-2 Production of Pullulans with Yeast Extract as a Nitrogen Source

The effect of concentration of yeast extract as a nitrogen source with 10% (w/v) sucrose as a carbon source on cell growth and production of pullulan was shown in Table 4. Cell growth increased with increased concentration of yeast extract whereas production of pullulan increased with a limited concentration of yeast extract. Also, the production of pullulan was enhanced up to a point by the concentration of yeast extract. The highest production of pullulan by *A. pullulans* HP-2001 was 39.95 g/l when the concentration of yeast extract as a nitrogen source was 0.25% (w/v) and its molecular weight was 1.26×10^6. The average molecular weight of pullulan ranged from 6.50×10^3 to 5.87×10^6 depending on the concentration of yeast extract. More than 0.25% (w/v) yeast extract in the medium showed negative effects for the production of pullulan.

Nitrogen depletion was essential for a higher production of pullulan as shown in some other exopolymers [58]. Yeast extract used as a nitrogen source is a complex mixture of

Table 4. Production of pullulan with 10% (w/v) sucrose and various concentration of yeast extract in a flask culture[a]

Yeast extract (%, w/v)	Final pH	DCW (g/l)	Pullulan (g/l)	$Y_{p/s}$	$Y_{p/x}$	Mw ($\times 10^4$)	Polydispersity (Mw/Mn)
0.00	2.91	4.00	10.95	0.11	2.74	0.7	1.02
0.05	2.88	9.30	16.00	0.16	1.72	587.0	3.61
0.15	2.96	13.05	29.10	0.29	2.23	325.6	4.12
0.20	2.98	13.40	32.35	0.32	2.41	245.2	3.85
0.25	3.25	15.10	39.95	0.40	2.65	125.7	3.20
0.30	3.44	15.90	34.05	0.34	2.14	107.1	2.97
0.35	3.71	17.10	28.65	0.29	1.68	101.8	2.87
0.50	3.96	19.60	20.75	0.21	1.06	115.8	3.10

a. Cultivated at 30℃, 200 rpm in a shaking incubator for 96 hr.

amino acids, peptides and protein, as well as a good source of vitamin B and some mineral salts [59,60]. It normally has a strong positive effect on bacterial growth rates [61], enzyme activity [62] and productivity of metabolites [63]. In the high concentrations of yeast extract as a nitrogen source, sugar was consumed mainly for the cell growth. In this respect, the concentrations of yeast extract can be used as one of the key factors for controlling the cell growth, productivity and molecular weight of pullulan. It seemed that a limited amount of yeast extract as a nitrogen source in the medium was essential for the production of pullulan by *A. pullulans* HP-2001 [57].

1-1-2-3 Effect of Initial pH on Production of Pullulan

The effect of the initial pH on production of pullulan was investigated by flask culture (Table 5). It has been noted that pH has profound effects on both the rate of production and the synthesis of extracellular polysaccharide [52,64,65]. So, it was of interest to investigate the effects of different initial pH values ranging from 3.0 to 8.0 in the medium on production of pullulan. The maximal cell growth of *A. pullulans* HP-2001 at a pH of 7.0 was achieved at the level of 15.10 g/l. However, the maximal production of pullulan was obtained at a pH 6.0, and that was 42.35 g/l, at which the total utilization rate of sucrose ($Y_{p/s}$ + $Y_{x/s}$) and specific yield (g pullulan/g DCW) were 0.56 and 3.00, respectively.

The pH of the culture broth was very important for cell growth, the production of pullulan and the morphological change of cells [52,65]. This result indicated that the optimal initial pH for the production of pullulan was different from that of cell growth [57].

Table 5. Effect of the initial pH on the production of pullulan in a flask culture[a]

Initial pH	Final pH	DCW (g/l)	Pullulan (g/l)	Yield		
				$Y_{p/s}$	$Y_{x/s}$	$Y_{p/x}$
3.00	2.18	10.80	30.20	0.30	0.11	2.80
4.00	2.39	11.45	32.40	0.32	0.11	2.83
5.00	2.50	12.95	36.75	0.37	0.13	2.83
6.00	3.68	14.10	42.35	0.42	0.14	3.00
7.00	3.87	15.10	39.95	0.40	0.15	2.64
8.00	3.83	12.55	36.00	0.36	0.13	2.87

a. Cultivated at 30℃, 200 rpm in a shaking incubator for 96 hr.

1-2. Effect of Agitation Speed on Production of Pullulan in a 7 liter Fermenter

1-2-1. Method

The effect of agitation speed on the production of pullulan in a 7 liter fermenter was investigated. The medium used for cell growth and the production of pullulan contained the following components (g/l): sucrose, 100; K_2HPO_4, 5.0; NaCl, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)_2SO_4$, 0.6 and yeast extract (Difco Lab., Detroit. USA), 2.5g. Agitation speed ranged from 300 to 600 rpm with an aeration rate of 1.0 vvm.

1-2-2. Results

The effect of agitation speed on the production of pullulan in a 7 liter fermenter was investigated (Fig. 4). Agitation speed ranged from 300 to 600 rpm with an aeration rate of 1.0 vvm. As the agitation speed of a 7 liter fermenter increased, the concentration of the dissolved oxygen in the medium increased during culture time and the period of exhaust of dissolved oxygen in the medium decreased (Fig. 4a). It seemed that a

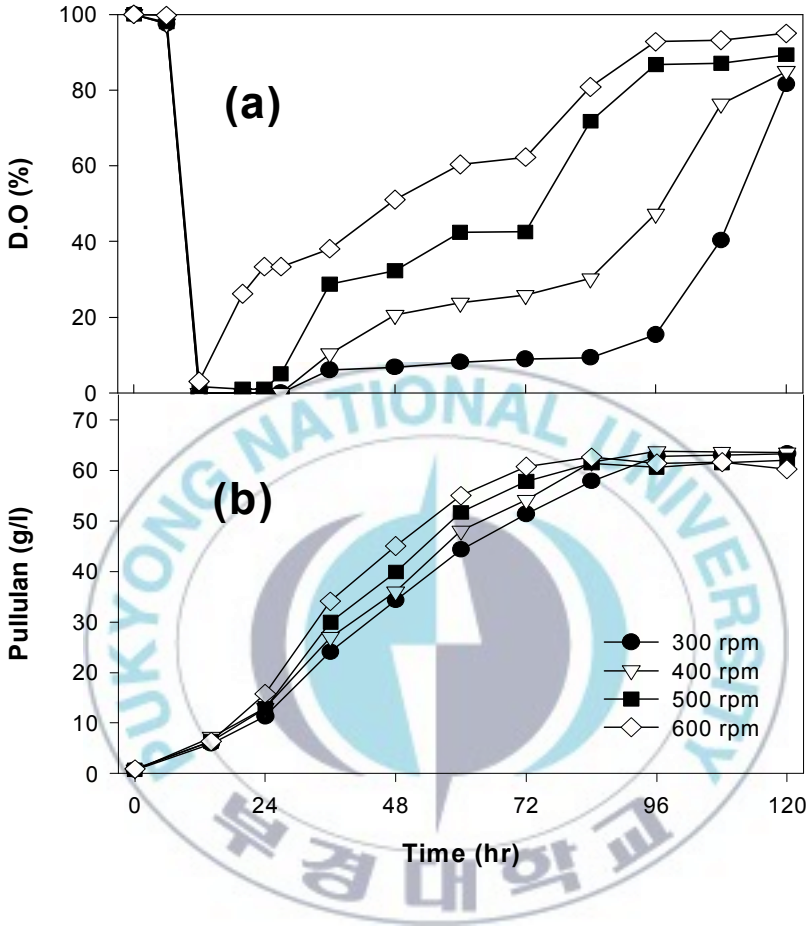


Fig. 4. Effect of agitation speed on the dissolved oxygen in the medium and production of pullulan. (a) Dissolved oxygen. (b) Production of pullulan. Cultivated at 30℃, 1.0 vvm and 5 liter working in a 7 liter fermenter for 120 hr.

higher agitation speed was able to maintain a relatively higher concentration of dissolved oxygen in the medium.

The productivity of pullulan with 600 rpm in a 7 liter fermenter was 0.844 g/l/h for 72 hr and the conversion rate of pullulan from 10% (w/v) sucrose was 60.8% (Fig. 4b). When the agitation rate was lower than 600 rpm, growth reached the stationary phase from 96 hr to 120 hr, and the productivity of pullulan diminished. Production of pullulan with a relatively high agitation speed was better than that with a low one. The maximal production of pullulan was obtained at the highest agitation speed (600 rpm) after only 72 hr, since further fermentation caused only a small increase in the production of pullulan and a decrease of productivity.

Batch culture for the production of pullulan by *A. pullulans* HP-2001 was performed in a 7liter fermenter (Fig. 5). Concentrations of sucrose and yeast extract in the medium were 10.0 and 0.25% (w/v), respectively, and initial pH adjusted 6.0. Cell growth and the production of pullulan in a 7 liter fermenter gradually increased with culture time. The pH in the medium decreased and then maintained around 3.2. The dissolved oxygen in the medium dramatically decreased during

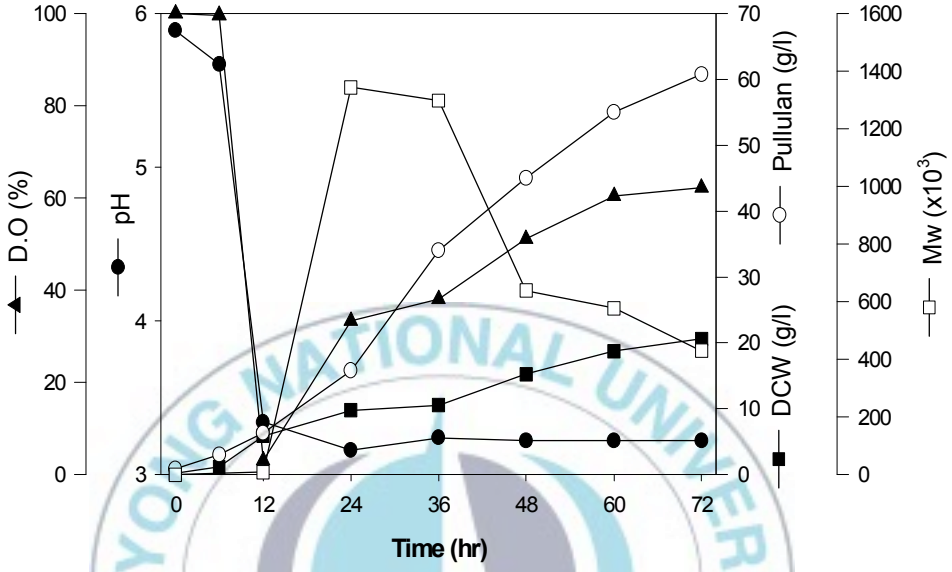


Fig. 5. Fermentation kinetics of *A. pullulans* HP-2001 in a 7 liter fermenter. The medium contained 10%(w/v) sucrose and 0.25% (w/v) yeast extract, initial pH was adjusted to 6.0. Cultivated at 30℃, 600 rpm, 1.0 vvm and 5 liter working in a 7 liter fermenter for 72 hr.

the log phase and then rose gradually around to 60%. Maximal production of pullulan by *A. pullulans* HP-2001 in a 7 liter fermenter reached 60.8 g/l after 72 hr. The molecular weight of pullulan increased until the middle of the log phase, then just as suddenly decreased until the stationary phase. The highest molecular weight of pullulans was 1.34×10^6, which was produced after 24 hr of culture. In the course of culture time, the molecular weight of pullulan decreased gradually.

The reduction in the amount of elaborated pullulan and its molecular weight might be due to the degradation of the polymer by the extracellular endoamylase in the late phase of culture [66]. Pullulans produced by *A. pullulans* had the same basic structures, but the ratios of their monomeric components were a little different [67], which might have been the reason for the production of pullulans with different molecular weights [68]. Due to the introduction of a small amount of mannose in the regular alternation of α-1,4 and α-1,6 bonds, pullulan produced may have been less sensitive to the α-amylase secreted into the medium, which resulted in the production of pullulans with higher molecular weights.

Pullulanase is one of the starch-debranching enzymes that

specifically attacks the branch points of amylopectin, hydrolyzing α-1,6 glucosidic linkages to produce maltotriose [69,70]. The increase in reducing sugar content after pullulanase treatment of pullulans produced after 24 hr and 72 hr of culture indicated that all the pullulans had α-1,6 glucosidic linkages of linked maltotriose units (Fig. 6). Higher percentage of reducing sugars in a pullulan produced after 72 hr than those 24 hr may have resulted from lower content of mannose in the pullulan. Also, the molecular weight of pullulan produced after 24 hr was higher than those produced after 72 hr. The exopolymers with increased mannose contents exhibited resistance to hydrolysis by α-amylase [67].

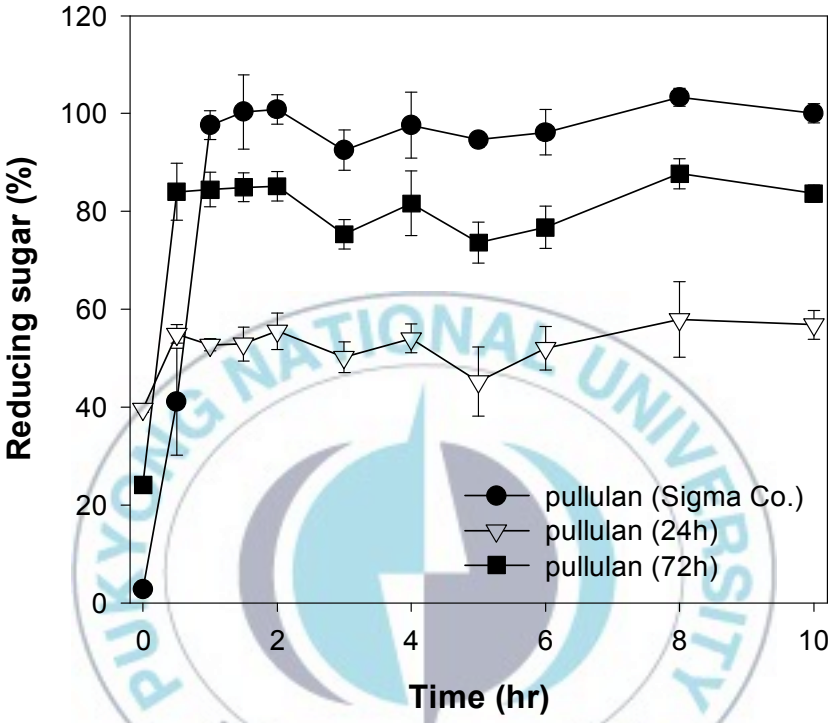


Fig. 6. Treatment of pullulan (Sigma Co.) and pullulans produced by *A. pullulans* HP-2001 for 24 hr and 72 hr with a pullulanase

1-3. Effect of Agitation Speed, Number of Impellers and Inner Pressure on the Production of pullulan in a 100 liter fermenter

1-3-1. Method

The effects of agitation speed on cell growth and production of pullulan in a 100 liter fermenter were investigated by changing the agitation speed from 150 to 350 rpm under a constant aeration rate of 1.0 vvm. The medium used for cell growth and the production of pullulan contained the following components (g/l): sucrose, 100; K_2HPO_4, 5.0; NaCl, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)_2SO_4$, 0.6 and yeast extract (Difco Lab., Detroit. USA), 2.5g.

To optimize the fermentation of *A. pullulans* HP-2001 in a 100 liter fermenter, the effect of the number of impellers on cell growth and production of pullulan was examined. Experiments employed a 100 liter fermenter (Ko-Biotech Co., Korea) with three six-bladed impellers and 3 baffles. The aeration rate, agitation speed and inner pressure were 1.0 vvm, 350 rpm and 0.4 kgf/cm^2.

The effect of inner pressure in a 100 liter fermenter on the production of pullulan and its molecular weight was investigated. The inner pressure of a 100 liter fermenter ranged from 0.0 to 0.8 kgf/cm^2. Agitation speed and aeration rate were 350 rpm and 1.0 vvm,

1-3-2. Results

1-3-2-1 Effect of Agitation Speed and Number of Impellers on the Production of Pullulan in a 100 liter fermenter

The effects of agitation speed on cell growth and production of pullulan in a 100liter fermenter were investigated by changing the agitation speed from 150 to 350 rpm under a constant aeration rate of 1.0 vvm (Fig. 7). Maximal production of pullulan and productivity were 32.12 g/l and 0.446 g/l/h, respectively, at 350 rpm and 1.0 vvm. Under the aeration rate of 1.0 vvm, the agitation speed was very important for the increase of productivity. With the increases of the aeration rate, the production of pullulan and productivity linearly increased form 8.75 to 26.50 g/l and from 0.122 to 0.368 g/l/h. An

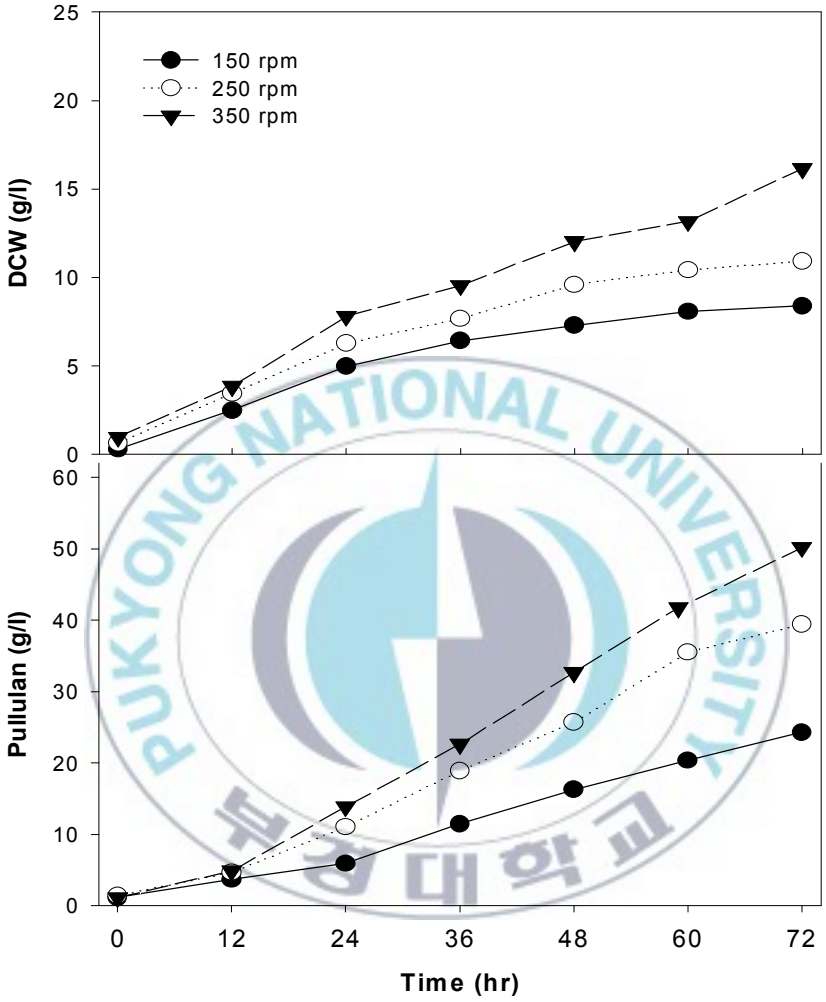


Fig. 7. Effect of agitation speed on cell growth and the production of pullulan in a 100 liter fermenter. Cultivated at 30℃, 1.0 vvm, 0.4 kgf/cm^2 and 70 liter working in a 100 liter fermenter for 120 hr.

agitation speed of 350 rpm and an aeration rate of 1.0 vvm were sufficient for the cell growth and production of pullulan. By increasing agitation speed, fermentation time was shortened and cell growth and production of pullulan were increased.

To optimize the fermentation of *A. pullulans* HP-2001 in a 100l fermenter, the effect of the number of impellers on cell growth and production of pullulan was examined (Fig. 8). Experiments employed a 100l fermenter (Ko-Biotech Co., Korea) with three six-bladed impellers and 6 baffles. The aeration rate, agitation speed and inner pressure were 1.0 vvm, 350 rpm and 0.4 kgf/cm^2, respectively, and working volume was 70 l in a 100 liter fermenter. More impellers can not contribute to the enhancement of the production of pullulan. Under sufficient agitation speed and aeration rate, a greater number of impellers showed to be useless for further cell growth and production of pullulan. However, the production of pullulan was greatly influenced by the agitation speed [67].

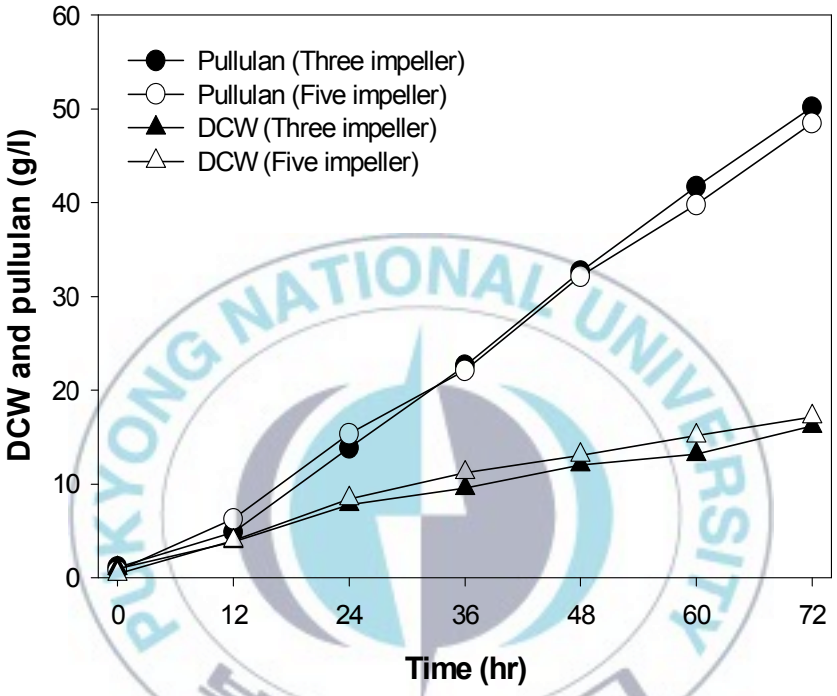


Fig. 8. Effect of the number of impellers on cell growth and the production of pullulan in a 100 liter fermenter. Cultivated at 30℃, 350 rpm, 1.0 vvm, 0.4 kgf/cm^2 and 70 liter working in a 100 liter fermenter for 72 hr.

1-3-2-2 Effect of the Inner Pressure on the Molecular Weight and Production of Pullulan in a 100 liter fermenter

The effect of inner pressure in a 100 liter fermenter on the production of pullulan and its molecular weight was investigated (Table 6). The inner pressure of a 100 liter fermenter ranged from 0.0 to 0.8 kgf/cm^2. Agitation speed and aeration rate were 350 rpm and 1.0 vvm, respectively. As the inner pressure of a 100 liter fermenter increased, the concentration of the dissolved oxygen in the medium increased during culture time and the time with a shortage of dissolved oxygen in the medium decreased (data not shown). Cell growth increased with increased inner pressure of a 100 liter fermenter. The molecular weight of pullulan decreased with increased inner pressure. Maximal production of pullulan was 56.53 g/l when the inner pressure of a 100 liter fermenter was 0.4 kgf/cm^2 at which the conversion rate and total utilization rate of sucrose ($Y_{p/s}$ + $Y_{x/s}$) were 0.57 and 0.73, respectively. The production of pullulan with an inner pressure of 0.4 kgf/cm^2 was 1.2 times higher than that without the inner pressure.

The inner pressure of a fermenter, which is one of physiological factors involved with the dissolved oxygen in the

Table 6. Effect of inner pressure on cell growth and production of pullulan[a]

Inner pressure (kgf/cm^2)	DCW (g/l)	Pullulan (g/l)	$Y_{p/s}$	$Y_{x/s}$	$Y_{p/x}$	productivity (g/l/h)
0.0	12.98	47.30	0.47	0.13	3.64	0.6569
0.2	13.52	48.83	0.49	0.14	3.61	0.6782
0.4	15.95	56.53	0.57	0.16	3.54	0.7851
0.6	17.83	50.60	0.51	0.18	2.83	0.7027
0.8	18.55	47.33	0.47	0.19	2.55	0.6574

a. The medium contained 10% (w/v) sucrose and 0.25% (w/v) yeast extract. Cultivated at 30℃, 350 rpm, 1.0 vvm and 70 liter working in a 100 liter fermenter for 72 hr.

medium, may affect molecular weight as well as production of pullulan (Fig. 9). It seems that higher inner pressure of a fermenter with a limited range to maintain a relatively high concentration of dissolved oxygen in the medium enhanced the production of pullulan by *A. pullulans* HP-2001 [67].

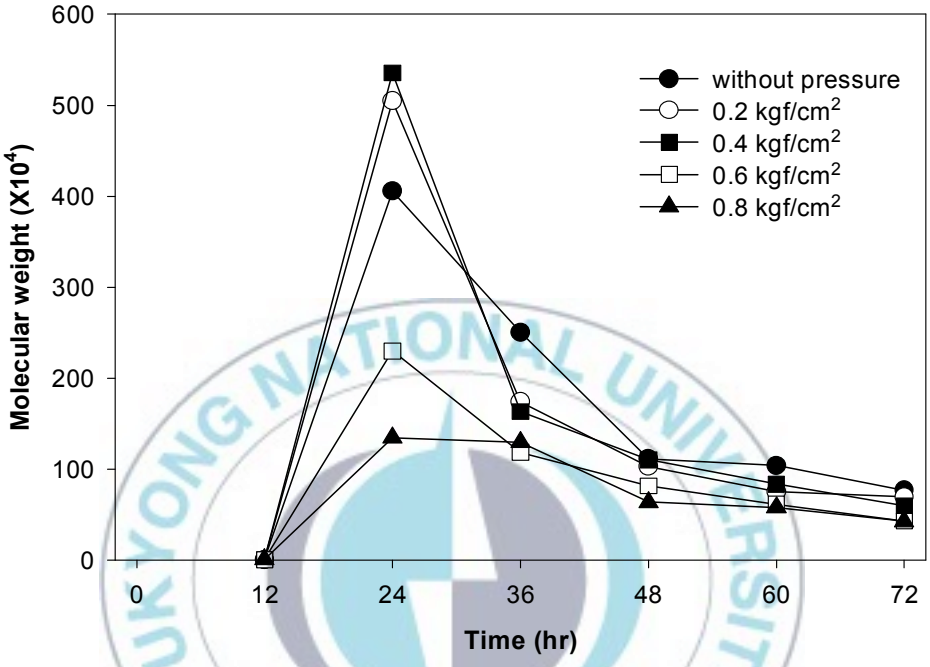


Fig. 9. Effect of inner pressure of a 100 liter fermenter on the molecular weight of pullulan. Cultivated at 350 rpm, 1.0 vvm and 70 liter working in a 100 liter fermenter for 72 hr.

1-4. Production of Pullulan in a 5 ton Fermenter

1-4-1. Method

The medium used for cell growth and the production of pullulan contained the following components (g/l): sucrose, 100; K_2HPO_4, 5.0; NaCl, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)_2SO_4$, 0.6 and yeast extract (Difco Lab., Detroit. USA), 2.5g. The inner pressure in the 5 ton was 0.4 MPa and aeration rate for the 5 ton was 1.0 vvm. Fermentations were incubated for 96 hours at 30℃.

1-4-2. Results

As shown in Fig. 10. cell growth increased with a 5 ton fermenter. Dissolved oxygen was decreased during the fermentation and decreased to about 0% at 24 hr. pH was decreased during 24 hr and maintained pH 3. Maximal production of pullulan by *A. pullulans* HP-2001 in a 5 ton fermenter with a inner pressure of 0.4 MPa was obtained with a molecular weight of 520 KDa.

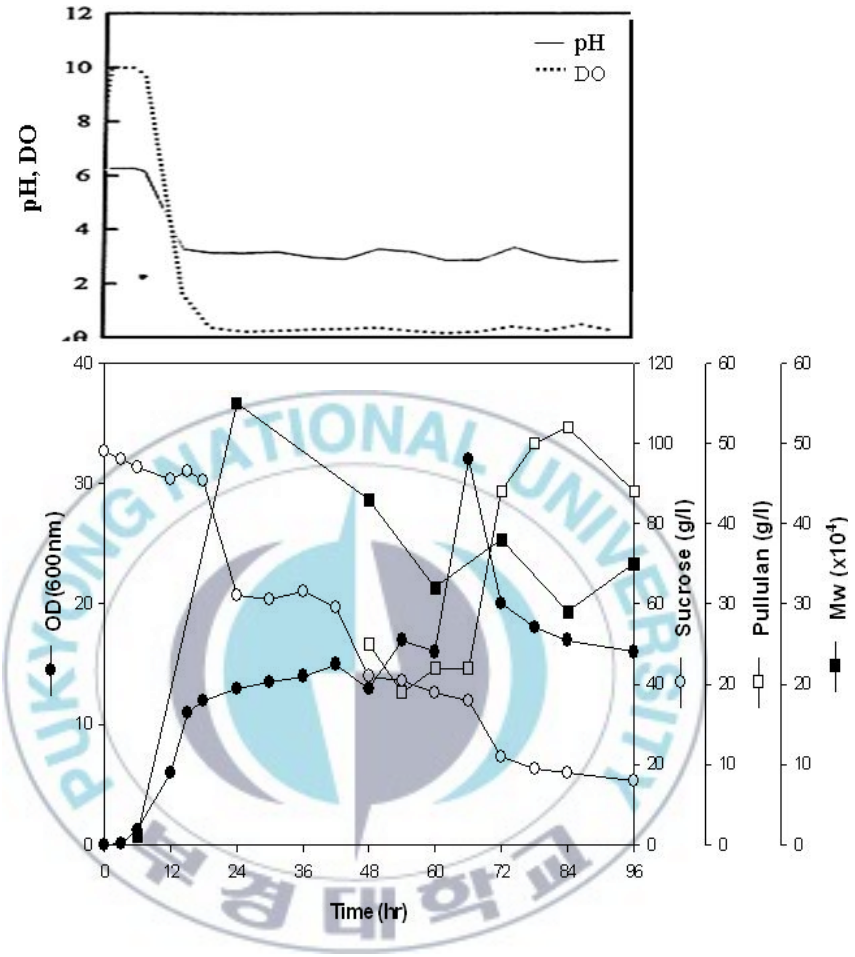


Fig. 10. Effect of a 5 ton fermenter on the molecular weight of pullulan. Cultivated 0.4 MPa, 1.0 vvm and 3 ton working in a 5 ton fermenter for 96 hr.

1-5. Production of Pullulan in a 6 ton Air-lift Type Fermenter

1-5-1. Method

The medium used for cell growth and the production of pullulan contained the following components (g/l): sucrose, 100; K_2HPO_4, 5.0; NaCl, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)_2SO_4$, 0.6 and yeast extract (Difco Lab., Detroit. USA), 2.5g. The fermentation for the production of Pullulan by *A. pullulans* HP-2001 was performed in 6 ton air-lift type fermenter. Working volumes of the 6 ton fermenter was 3 ton, respectively. Temperature for fermentation with 6 ton fermenter was maintained at 30℃. Air lift type in which no mechanical stirrers are used and the agitation is achieved by the air bubbles generated by the air supply. The aeration rate for the 6 ton fermenter was 1.0 kg/cm^3. The fermentation was incubated for 65 hr.

1-5-2. Results

The fermentation were investigated with an airlift fermenter system in a 6 ton. The aeration rate was 1.0 kg/cm^3 at the initial pH 6.0. Dissolved oxygen was decreased during the

fermentation and decreased to about 60% for 12 hr. Dissolved oxygen was a little increased during fermentation. Maximal production of pullulan by *A. pullulans* HP-2001 in a 6 ton fermenter was 48.80 g/l of pullulan(Fig. 11).

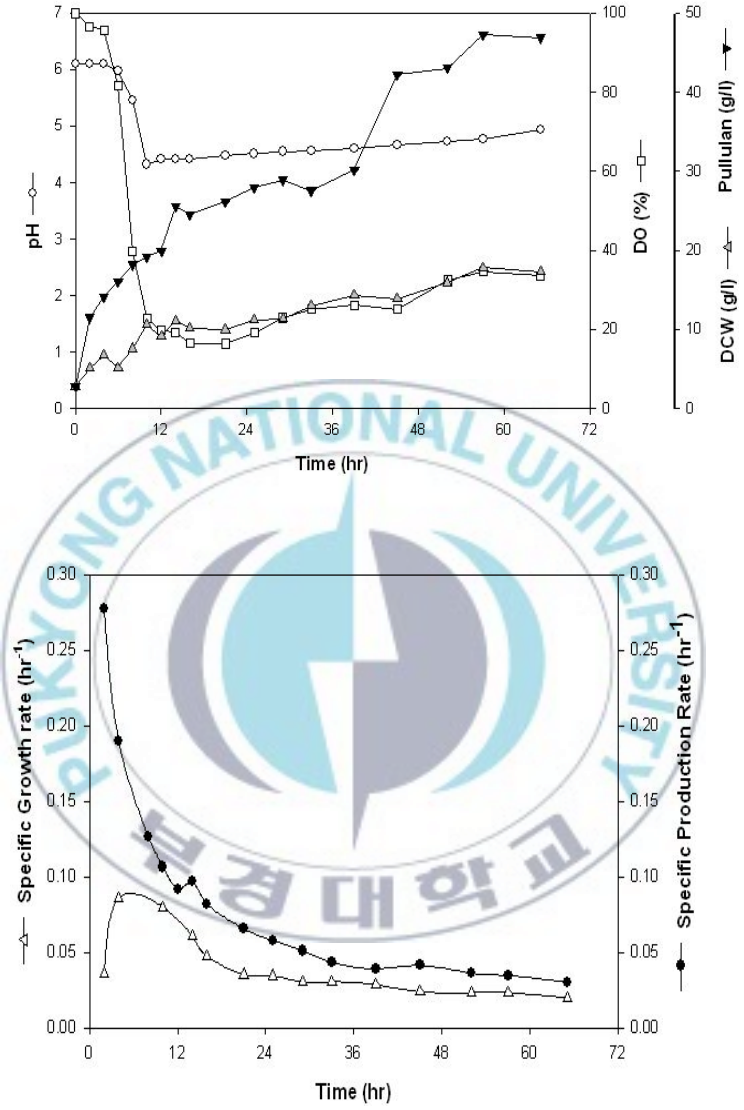


Fig. 11. Production of pullulan in a 6 ton air-lift type fermenter

2. Production of Pullulan by *Aureobasidium pullulans* HP-2001 with Continuous Fermentation

2-1. Effect of Medium Repeated Fed Batch on the Production of Pullulan

2-1-1. Method

The general growth kinetic of batch culture of *A. pullulans* HP-2001 in a 7 liter fermenter was shown in Fig. 12. The medium contained (g/l) sucrose, 10; and yeast extract, 2.5; K_2HPO_4, 5.0; NaCl, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)_2SO_4$, 0.6. The aeration rate and agitation speed were 1.0 vvm and 500 rpm.

The volume of substituted solution was half of the original working volume. Substituted solutions used in this study were 1) 10% (w/v) sucrose, 2) 10% (w/v) sucrose and 0.25% (w/v) yeast extract and 3) 10% (w/v) sucrose, 0.25% (w/v) yeast extract and mineral salts, which is the medium for the production of pullulan. The mineral salts were composed of 5.0 g/l K_2HPO_4, 1.0 g/l NaCl, 0.2 g/l $MgSO_4 \cdot 7H_2O$ and 0.6 g/l $(NH_4)_2SO_4$.

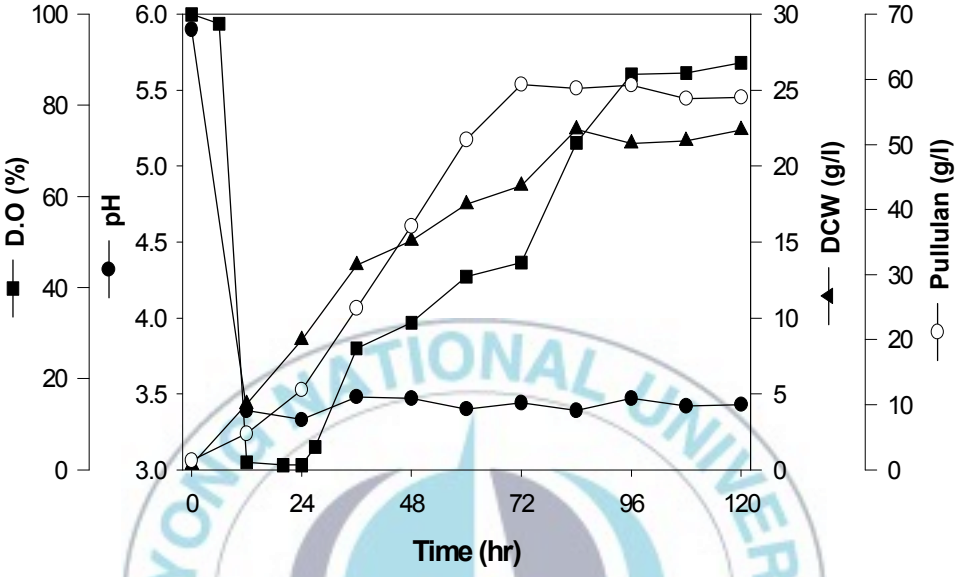


Fig. 12. Cell growth and production of pullulan by *A. pullulans* HP-2001 with 10% (w/v) sucrose and 0.25% (w/v) yeast extract in a 7 liter fermenter. Cultivated at 30℃, 500 rpm, 1.0 vvm and 5 liter working in a 7 liter fermenter for 120 hr

2-1-2. Results

The effect of medium repeated fed batch after 72 hr on cell growth and the production of pullulan by *A. pullulans* HP-2001 was examined in a 7 liter fermenter (Fig. 13). The volume of substituted solution was half of the original working volume. Substituted solutions used in this study were 1) 10% (w/v) sucrose, 2) 10% (w/v) sucrose and 0.25% (w/v) yeast extract and 3) 10% (w/v) sucrose, 0.25% (w/v) yeast extract and mineral salts, which is the medium for the production of pullulan. The mineral salts were composed of 5.0 g/l K_2HPO_4, 1.0 g/l NaCl, 0.2 g/l $MgSO_4 \cdot 7H_2O$ and 0.6 g/l $(NH_4)_2SO_4$. The pH of the substituted solution were adjusted to 6.0 before sterilization. When the substituted medium contained 10% (w/v) sucrose, 0.25% (w/v) yeast extract and mineral salts, the maximal production of pullulan was 75.88 g/l at 120 hr. After 72 hr repeated fed batch, dissolved oxygen was almost exhausted. Overall production of pullulan fed with fresh medium was about 1.80 times higher than those of pullulan by batch culture.

When the substituted medium contained only 10% (w/v) sucrose, the concentration of pullulan and productivity were

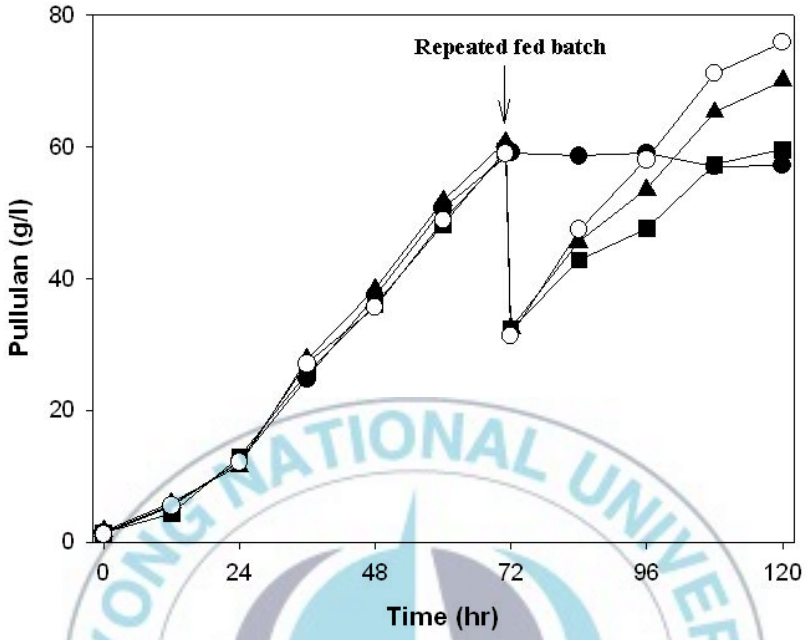


Fig. 13. Effect of medium repeated fed batch after 72 hr on the production of pullulan by *A. pullulans* HP-2001: batch culture without repeated fed batch (●); repeated fed batch solution containing 10%(w/v) sucrose (■); 10% (w/v) sucrose and 0.25% (w/v) yeast extract (▲); 10% (w/v) sucrose, 0.25% (w/v) yeast extract and mineral salts (○). Cultivated at 30℃, 500 rpm, 1.0 vvm, 5 liter working in a 7 liter fermenter for 120 hr

59.63 g/l and 0.50 g/l/h, respectively, at 120 hr. The final concentration of pullulan in the medium was similar to that of batch culture but overall production of pullulan was 1.56 times higher than that of pullulan by batch culture. Productivity of pullulan fed with the fresh medium was higher than that fed with the medium containing 10% (w/v) sucrose, 10% (w/v) sucrose and 0.25% (w/v) yeast extract. Anyway, feeding nutrient to the fermenter at steady-state was considered to be a beneficial method for the higher production of pullulan by *A. pullulans* HP-2001 [67].

2-2. Production of Pullulan in Continuous Culture and Varying Feed Concentration of Sucrose in Continuous fermentation

2-2-1. Method

Continuous culture of *A. pullulans* HP-2001 was performed in a 7 liter fermenter and the effect of dilution rate on cell growth and the production of pullulan was examined. The aeration rate and agitation speed were 1.0 vvm and 500 rpm, respectively. The feed solution was the fresh medium containing sucrose, yeast extract and mineral salts. The medium contained (g/l) sucrose, 10; and yeast extract, 2.5; K_2HPO_4, 5.0; NaCl, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)_2SO_4$, 0.6. The continuous medium feed was commenced after 72 hr of batch fermentation. The concentration of pullulan at steady-state increased with increased dilution rate up to 0.015 h^{-1} and that was 75.0 g/l. Above a dilution rate of 0.015 h^{-1}, steady-state could not be maintained and a wash out of cells occurred. These results indicated that optimal dilution rate for the production of pullulan was 0.015 h^{-1} in the continuous culture of *A. pullulans*

HP-2001.

A. pullulans HP-2001 was grown in continuous culture at constant dilution rate (D=0.015 h^{-1}) with varying feed concentrations of sucrose (10-20%, w/v) as the carbon source. The aeration rate and agitation speed were 1.0 vvm and 500 rpm, respectively. When the feed solution contained 20% (w/v) sucrose, 0.25% yeast extract and mineral salts, above 100 g/l of pullulans were obtained at steady-state

2-2-2. Results

Continuous culture of *A. pullulans* HP-2001 was performed in a 7 liter fermenter and the effect of dilution rate on cell growth and the production of pullulan was examined (Fig. 14). The aeration rate and agitation speed were 1.0 vvm and 500 rpm, respectively. The feed solution was the fresh medium containing sucrose, yeast extract and mineral salts. The continuous medium feed was commenced after 72 hr of batch fermentation. The concentration of pullulan at steady-state increased with increased dilution rate up to 0.015 h^{-1} and that was 75.0 g/l. Above a dilution rate of 0.015 h^{-1}, steady-state could not be maintained and a wash out of cells occurred. These results indicated that optimal dilution rate for the

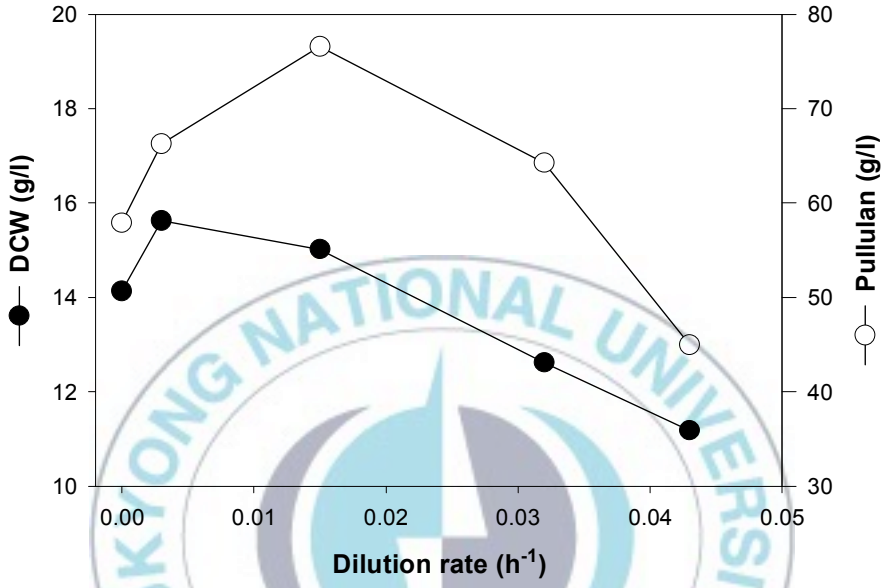


Fig. 14. Effect of dilution rate on the production of pullulan by continuous culture. Extrapolated values for zero dilution rate obtained from batch fermentation.

production of pullulan was 0.015 h^{-1} in the continuous culture of *A. pullulans* HP-2001.

Distribution of molecular weights of pullulan with a dilution rate of 0.015 h^{-1} was shown Fig. 15. The molecular weight of pullulan increased until the middle of the log phase, then just as suddenly decreased until the stationary phase. The highest Mw and Mn of pullulan were 3.52×10^6 and 1.22×10^6, respectively, which was produced after 24 hr of culture, then just as suddenly decreased. After feeding fresh medium, the Mw and Mn of pullulan slightly increased to around 5.40×10^5 and 1.25×10^5. The narrowest polydispersity (Mw/Mn) of pullulan was 2.35 at 48 hr and polydispersity increased around 4.40 with culture time. Above 70.0 g/l of pullulan and 4.0×10^5 of Mw of the products were obtained after feeding. Unlike previous reports that average molecular weights of pullulan decreased late in the stationary phase due to the presence of the amylase secreted into the medium [71], the decline of Mw and Mn was not found in the continuous culture of *A. pullulans* HP-2001. These results suggested that variation of molecular weight of pullulan by continuous culture may be related with the presence of pullulanase. It was supposed that feeding nutrient to fermenter extended steady-state and retarded the excretion of pullulanase.

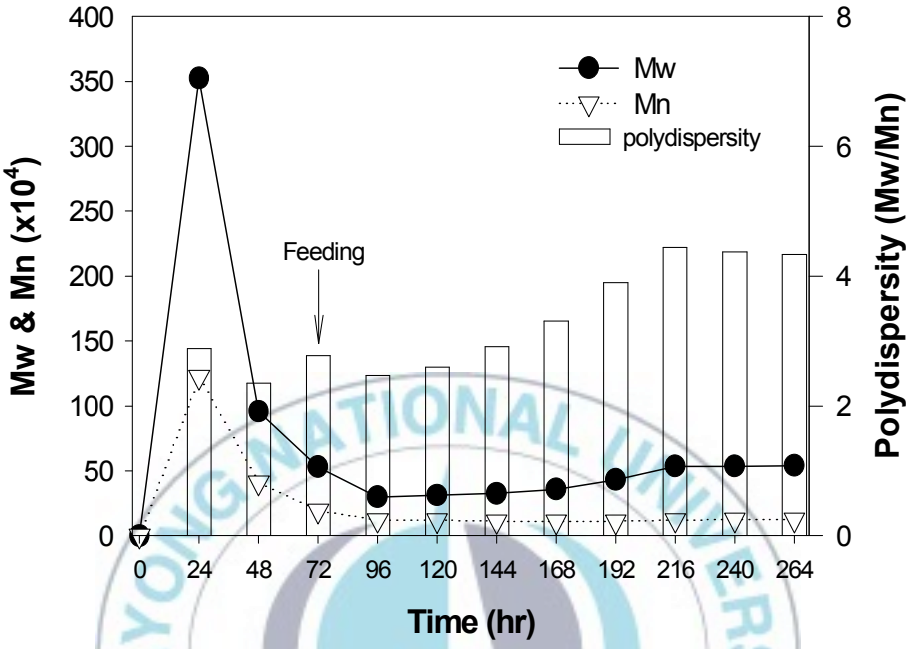


Fig. 15. Distribution of Mw and Mn of pullulan produced by continuous culture with a dilution rate of 0.015 h^{-1}. Cultivated at 30℃, 500 rpm, 1.0 vvm, 5 liter working in a 7 liter fermenter for 264 hr.

A. pullulans HP-2001 was grown in continuous culture at constant dilution rate (D=0.015 h^{-1}) with varying feed concentrations of sucrose (10-20%, w/v) as the carbon source (Fig. 16). The aeration rate and agitation speed were 1.0 vvm and 500 rpm, respectively. When the feed solution contained 20% (w/v) sucrose, 0.25% yeast extract and mineral salts, above 100 g/l of pullulans were obtained at steady-state. Steady-state in the continuous culture fed with feed solution containing 20% (w/v) sucrose maintained longer than that fed with feed solution containing 10% (w/v) sucrose. When feed solution contained more than 10% (w/v) sucrose, a higher concentration of pullulan was obtained at steady-state. Under optimal dilution rate, *A. pullulans* HP-2001 seemed overcome the catabolite repression up to 20% (w/v) sucrose in the feed solution [67].

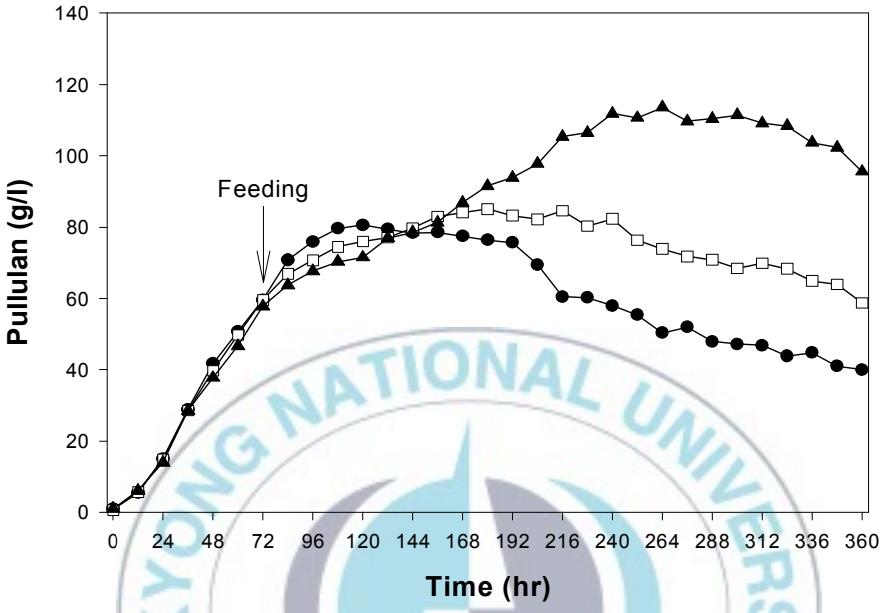


Fig. 16. Effect of sucrose concentration in the feed solution on the production of pullulan by continuous culture. The feed concentration of sucrose were 10% (●), 15% (□) and 20% (▲)(w/v), respectively. Cultivated at 30℃, 500 rpm, 1.0 vvm, 5 liter working in a 7 liter fermenter with a dilution rate of 0.015 h^{-1} for 360 hr.

3. Control of Pullulan Molecular Weight with Variation of Culture Conditions

3-1. Variation Tendency of Pullulan Molecular Weight during Fermentation Process

3-1-1. Method

The medium used for cell growth and the production of pullulan contained the following components (g/l): sucrose, 100; K_2HPO_4, 5.0; NaCl, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)_2SO_4$, 0.6 and yeast extract (Difco Lab., Detroit. USA), 2.5 [51]. Cultures were grown initially in 200 ml medium in 500 ml flasks for 3 days and shaken at 200 rpm. The resulting cultures were incubated for 3 days at 30℃ under aerobic condition. The initial pH and agitation speed on culture broth were controlled at 6.8 respectively.

3-1-2. Results

The objectives for this study was to evaluate for the production of pullulan with molecular weight in a fermentation. The high molecular weight of pullulan increased in the initial

culture early. After the stationary phase decreased the molecular weight of pullulan(Fig. 17).

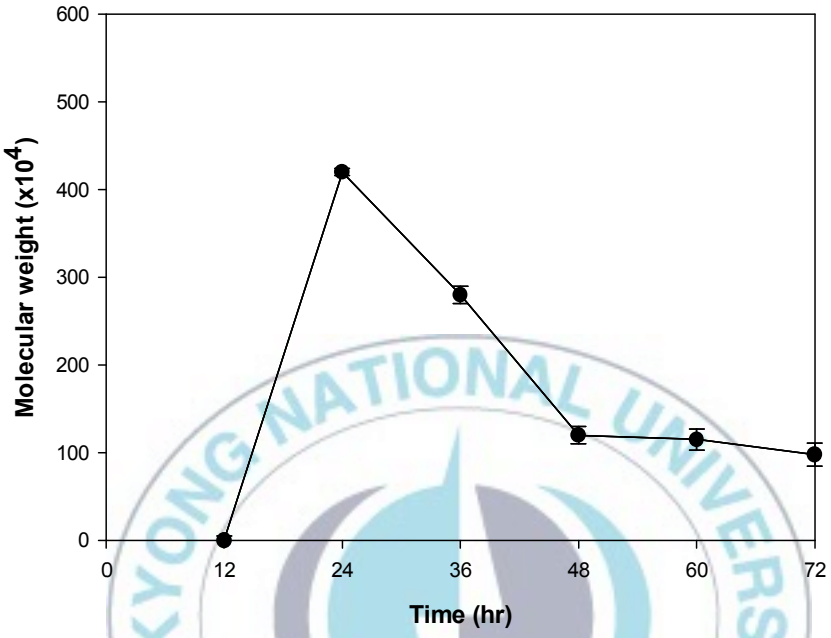


Fig. 17. Molecular weight of pullulan produced in a 7 liter fermenter

3-2. Effect of Initial pH on the Molecular Weight of Pullulan in a Fermentation

3-2-1. Method

The medium used for cell growth and the production of pullulan contained the following components (g/l): sucrose, 100; K_2HPO_4, 5.0; NaCl, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)_2SO_4$, 0.6 and yeast extract (Difco Lab., Detroit. USA), 2.5 [51]. Cultures were grown initially in 200 ml medium in 500 ml flasks for 3 days and shaken at 200 rpm. The resulting cultures were incubated for 3 days at 30℃ under aerobic condition. The initial pH and agitation speed on culture broth were controlled at 6.8 respectively. Pullulan production from *A. pullulans* HP-2001 were determined at various culture conditions with pH as variables from pH 3 to 8. The medium pH was adjusted to 3 to 8 in steps of 1.0 pH unit by adding 1 N NaOH or 1 N HCl prior to sterilization.

3-2-2. Results

Important parameters for pullulan synthesis are temperature,

pH of the medium, oxygen supply, nitrogen concentration, and carbon source [72,73,74]. Among these parameters, the pH of the culture broth is one of the main factors influencing the production of pullulan. The yield of pullulan production, the important parameter for the commercial application of pullulan is the molecular weight. Pullulan with a high molecular weight has a high viscosity, thus, it is more valuable than that with a low molecular weight.

The pH effects are often investigated using the same microorganism in flask experiments with different initial pH values. The maximum production of pullulan with an initial pH 5 was achieved at the level of 47.76 g/l. The pullulan production increased from initial pH 3 to 5. However, a high proportion of high molecular weight pullulan (M.W 1.64 x 10^6) was produced at a pH of 3. Pullulan degrading enzyme was activated when the pH of the broth was lower than 5.0 and the portion of low molecular weight pullulan was increased. The reson for the decrease in the high molecular weight portion of pullulan at low pH is due to the pullulanase activation at a low pH and acid hydrolysis at low pH conditions [75](Fig. 18).

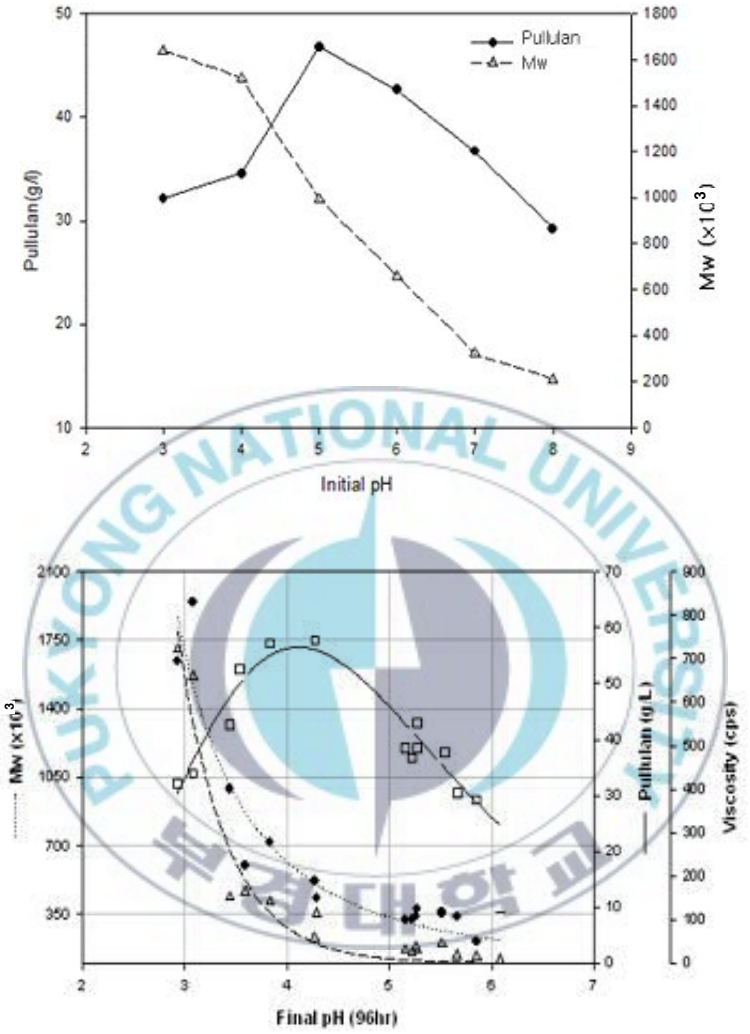


Fig. 18. Effect of initial pH on the molecular weight of pullulan

3-3. Two-stage Fermentation Process for Optimal Production of Pullulan

3-3-1. Method

The medium used for cell growth and the production of pullulan contained the following components (g/l): sucrose, 100; K_2HPO_4, 5.0; NaCl, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)_2SO_4$, 0.6 and yeast extract (Difco Lab., Detroit. USA), 2.5 [51]. The effect of pH fermentation was studied by batch fermentation in a 7 liter fermenter with a pH control. The fermentation with 4 liter of medium was operated at temperature 30℃, 1 vvm aeration and agitation 500 rpm for about 96 hr. Two-stage fermentation was demonstrated by controlling the culture pH at 4.0 around 24, 48, 60 hr for cell growth. It was the shifted to pH 5.0 for production of pullulan in the secondary stage.

3-3-2. Results

Two-stage pH operation was performed to optimize production of pullulan as demonstrated in Fig. 19. The experiment of result, in the first stage, the fermentation pH

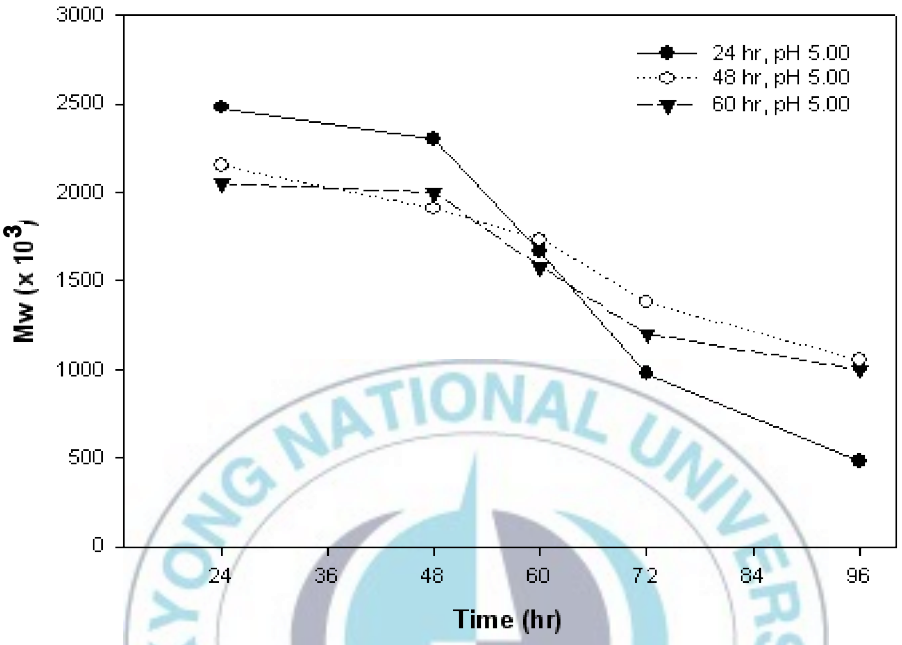


Fig. 19 . Two-stage fermentation with culture pH shifted from pH 4.0 to 5.0 after 24, 48 ,60 hr of cultivation

controlled at pH 4.0 around 24 hr for cell growth and it was then shifted to pH 5.0 production of pullulan in the secondary stage, The two-stage fermentation process that maximized product formation was demonstrated with a molecular weight changes were achieved [76].

3-4. The Effect of $CaCO_3$ and Ascorbic Acid Concentration on the Molecular Weight of Pullulan

3-4-1. Method

The effect of $CaCO_3$ concentration in culture was studied using shake flask cultures at different concentration of $CaCO_3$. The medium contained (g/l) sucrose, 10; and yeast extract, 2.5; K_2HPO_4, 5.0; NaCl, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)_2SO_4$, 0.6. The concentration of $CaCO_3$ ranged from 0.0 to 20 g/l. The culture was incubated on a rotary shaker at 30℃ and 200 rpm for 72 hr, 96 hr.

The effect of ascorbic acid concentration in culture was studied using shake flask cultures at different concentration of ascorbic acid . The medium contained (g/l) sucrose, 10; and yeast extract, 2.5; K_2HPO_4, 5.0; NaCl, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)_2SO_4$, 0.6. The concentration of ascorbic acid ranged from 0.0 to 20 g/l. The culture was incubated on a rotary shaker at 30℃ and 200 rpm for 72 hr. The concentration of ascorbic acid ranged from 0.0 to 3 g/l.

3-4-2. Results

The pH is kept constant during the production process than to put a buffer that is easy to cultivate. So in this study, the effect of $CaCO_3$ concentration containing buffer capacity on production of pullulan. In shake flasks, addition of $CaCO_3$ and $CaCO_3$ concentration ranged from 0.0 to 20 g/l. The production of molecular weight of pullulan with below 1 g/l of $CaCO_3$ was decreased . A high proportion of high molecular weight pullulan (M.W 1.0 x 10^6) was added 0.1 g/l of $CaCO_3$(Fig. 20, 21).

A microbial fermentation and recovery process was developed for large-scale production. One of undesirable features of fermentation of *A. pullulans* are readily observed. The problem is the simultaneous synthesis of dark melanin-like pigment, which contaminates the pullulan during long culture time. A decolorization process with added ascorbic acid during fermentation to remove the pigment. Ascorbic acid is well known that ascorbic acid plays the role of an effective antioxidant in biological systems [77] and to remove the pigment and to contain buffer capacity. Therefore, it attracts some interest in studies of the influence of ascorbic acid on the

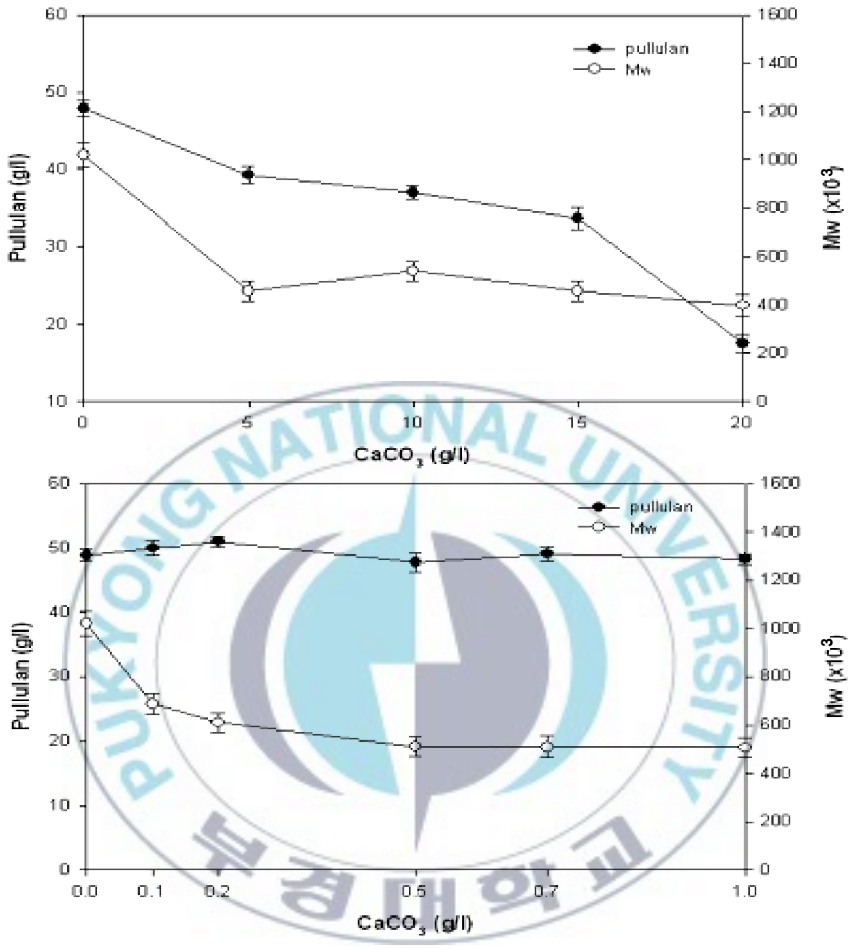


Fig. 20. Effect of concentration of $CaCO_3$ on production of pullulan and on the molecular weight of pullulan by *A. pullulans* HP-2001(72 hr)

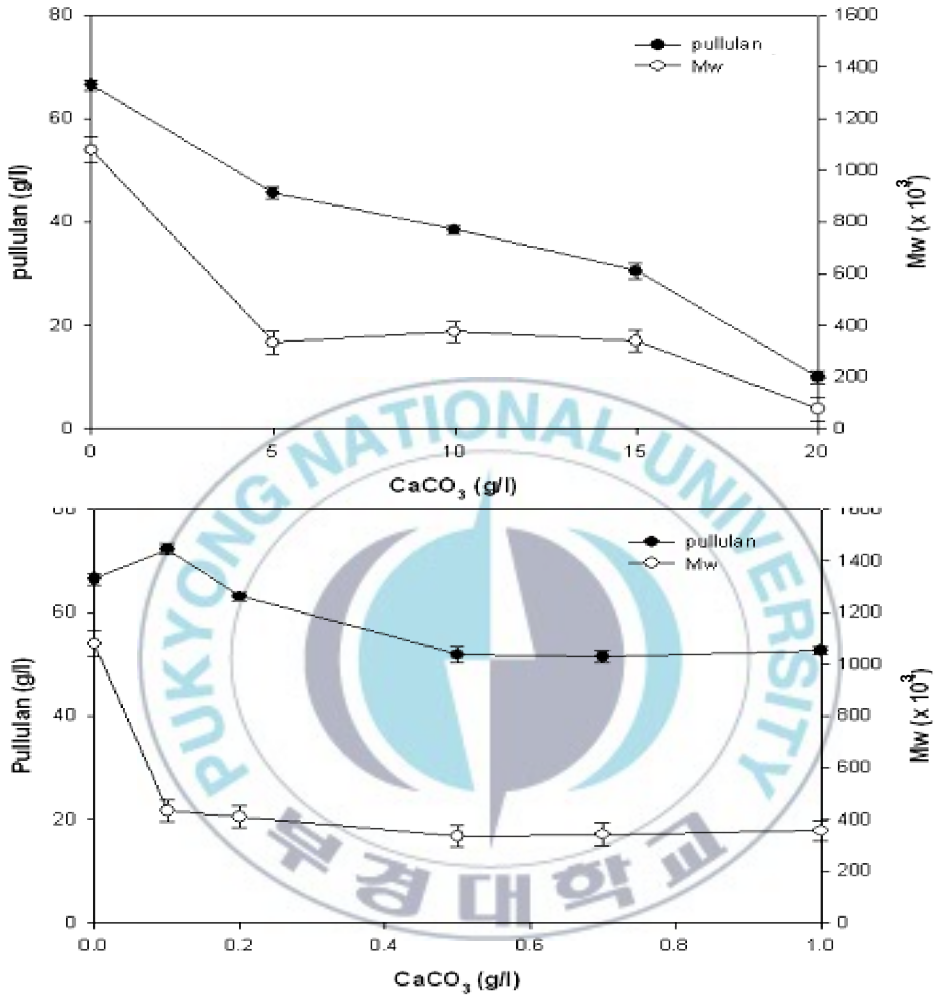


Fig. 21. Effect of concentration of $CaCO_3$ on production of pullulan and on the molecular weight of pullulan by *A. pullulans* HP-2001(96 hr)

stability of pullulan in ascorbic acid-containing systems as well as influence of pullulan on the decomposition rate of ascorbic acid in solution. The concentration of ascorbic acid ranged from 0.0 to 3 g/l. The maximum concentration of ascorbic acid was 1 g/l (Fig. 22).

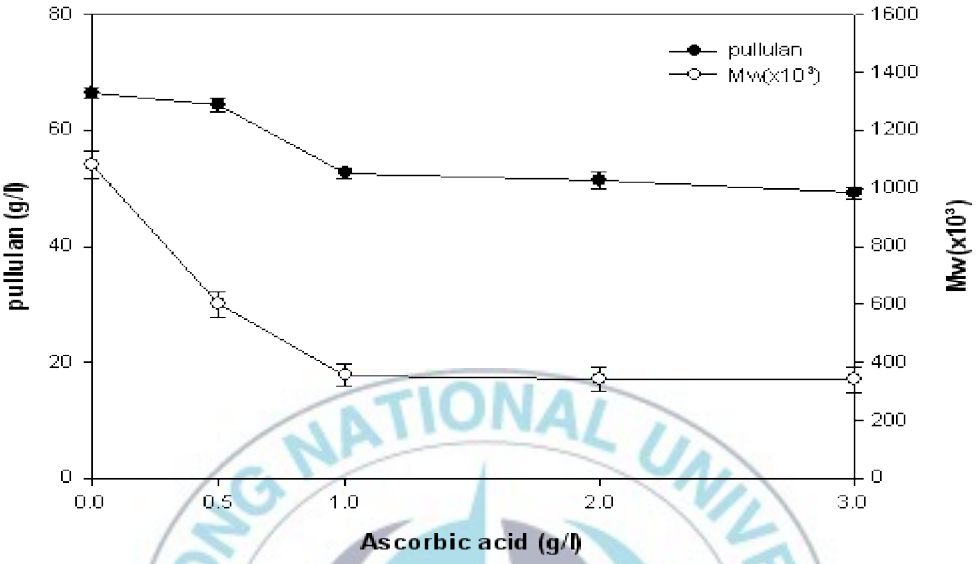


Fig. 22. Effect of concentration of Ascorbic acid on production of pullulan and molecular weight of pullulan by *A. pullulans* HP-2001.

3-5. Effect of Hydrolysis Reaction on the Molecular Weight of Pullulan

3-5-1. Method

Fermentation for the production of pullulan were performed in 500 liter fermenter.(Ko-Biotech Co.,Korea). Working volumes of the 500 liter fermenter was 300liter. The medium used for cell growth and the production of pullulan contained the following components (g/l): sucrose, 100; K_2HPO_4, 5.0; NaCl, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)_2SO_4$, 0.6 and yeast extract (Difco Lab., Detroit. USA), 2.5 [51]. The agitation speed was maintained at 150 rpm, and aeration rate was 0.5 vvm. The inner pressure in the 500 liter fermenter was 0.2 kgf/cm^2.

1 M H_2SO_4 was added to culture solution for 3 hours to get lower molecular weight of pullulan.

3-5-2. Results

The viscosity of the fermentation increased with a hig molecular weight (Fig. 23). Specically the molecular weight control which leads the hydrolysis of pullulan. The pullulan low

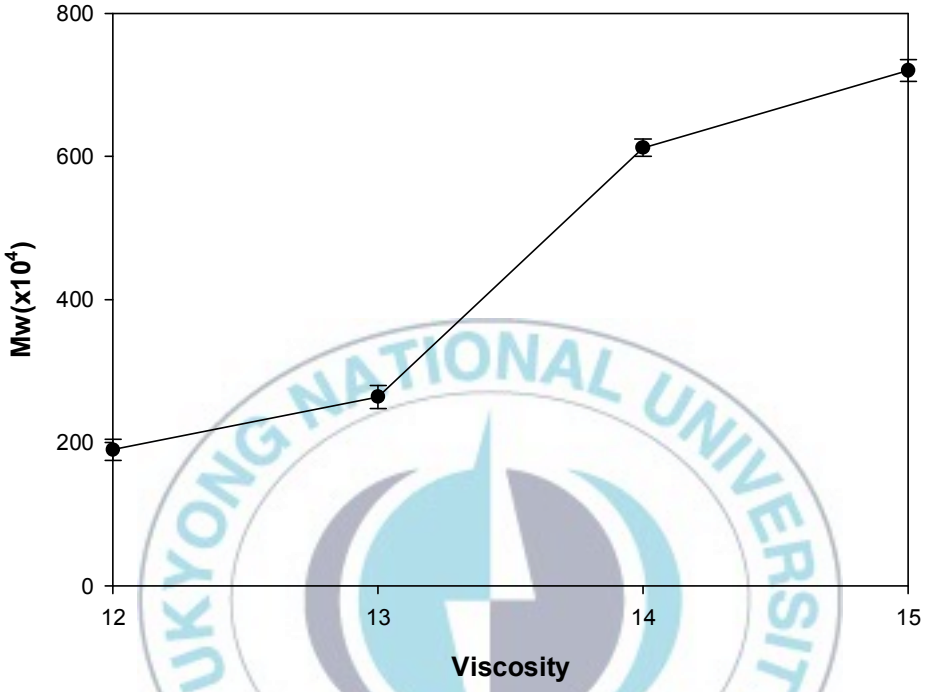


Fig. 23 . Effect of a various viscosity on molecular weight by *A. pullulans* HP-2001

molecular weight increased after 1 - 3 hours treated with 1 M H_2SO_4. After 3 hour of molecular weigh was 2.84 x 10^5 . The reason for the decrease in the high molecular weight of pullulan acid hydrolysis at low pH conditions (Table 7.)

Table 7. Effect of acid hydrolysis on production of pullulan and control of molecular weight by *A. pullulans* HP-2001.

acid hydrolysis (hr)	Viscosity (cps 25℃)		pullulan	Mw ($x10^3$)
	initial	final		
0	14	14	25.2	609
1	14	12.7	27.0	340
2	14	12.3	26.8	301
3	14	13	27.2	284

3-6. Effect of Substrate of Medium on the Molecular Weight of Pullulan

3-6-1. Method

The medium used for cell growth and the production of pullulan contained the following components (g/l): sucrose, 100; K_2HPO_4, 5.0; NaCl, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)_2SO_4$, 0.6 and isolated soybean protein (ISP) 0-1.0%. The ISP used as a nitrogen source in this study was the agro-industrial byproduct. It consisted of the following components (%): crude protein, 45, 50; crude cellulose 5-6.

3-6-2. Results

The average molecular weights of pullulans produced with various concentrations of ISP, ranged from 1.34 to 5.88 x $10^{6.}$ Pullulans produced with ISP , the molecular weight increased as was added ISP, reaching its maximum when the concentration of ISP was about 0.45%. Overall the molecular weights of pullulans produced with was ISP 4 times higher than those of pullulans produced with yeast extract as a nitrogen source.

The average molecular weights of pullulans ranged from 1.5×10^4 to 1.0×10^7 depending on culture conditions and stains [50,51,78](Fig. 24). The molecular weights of exopolymers produced with agricultural wastes were higher than those produced with glucose as a carbon source [79]. Substrates for the production of pullulan and the initial pH of the medium also affected the molecular weight of the pullulan [80, 81].

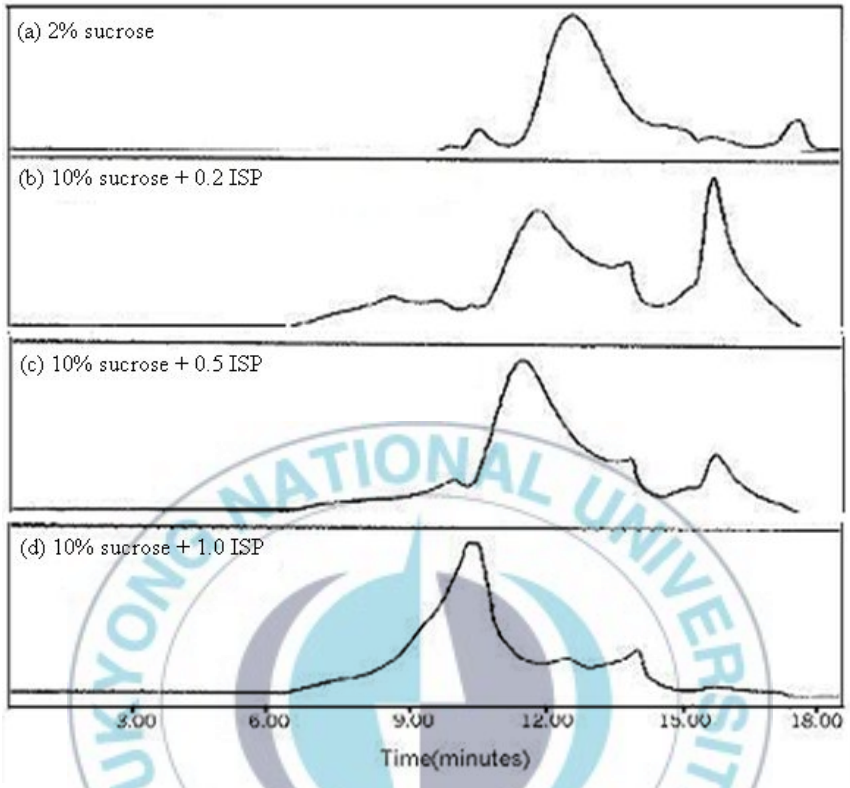


Fig. 24. GPC chromatograms of pullulans made with ISP as a nitrogen sources. (a); 2 % sucrose (b); 10 % sucrose, 0.2 % ISP (c); 10% sucrose, 0.5 % ISP (d); 10 % sucrose, 1.0 ISP

3-7. Optimum Condition of Pullulan Production with Specific Molecular Weight by *A. pullulans* HP-2001

For the production of specific molecular weight pullulans with 1000KD, 500KD and 300KD, specific conditions for the fermentations were determined as shown below The yeast extract concentrations and pH of the fermentation broth were controlled to produce specific pullulan with desired molecular weights.

① Conditions of 1,000 KD molecualr weight pullulan by *A. pullulans* HP-2001

Medium	g/l	Conditions	
K_2HPO_4	5.0	Agitation speed (rpm)	80
NaCl	1.0	Culture time (hr)	60
$MgSO_47H_2O$	0.2	Inoculum size(%)	3
$(NH_4)_2SO_4$	0.6	Temperature (℃)	30
yeast extract or ISP	>2.5	Inner pressure (kg/cm^2)	0.5
		Initial pH	6.0
Sucrose	100	Aeration rate (LPM)	1500

② Conditions of 500 KD molecualr weight pullulan by *A. pullulans* HP-2001

Ⅰ.

Medium	g/l	Conditions	
K_2HPO_4	5.0	Agitation speed (rpm)	80
NaCl	1.0	Culture time (hr)	60
$MgSO_47H_2O$	0.2	Inoculum size(%)	3
$(NH_4)_2SO_4$	0.6	Temperature (℃)	30
yeast extract	2.5	Inner pressure (kg/cm^2)	0.5
$CaCO_3$	**0.5**	Initial pH	**6.0**
Sucrose	100	Aeration rate (LPM)	1500

Ⅱ.

Medium	g/l	Condition	
K_2HPO_4	5.0	Agitation speed (rpm)	80
NaCl	1.0	Culture time (hr)	60
$MgSO_47H_2O$	0.2	Inoculum size(%)	3
$(NH_4)_2SO_4$	0.6	Temperature (℃)	30
yeast extract	2.5	Inner pressure (kg/cm^2)	0.5
$CaCO_3$	**0.25**	Initial pH	**6.0**
Ascorbic acid	**1**		
Sucrose	100	Aeration rate (LPM)	1500

③ Conditions of 300 KD molecualr weight pullulan by *A. pullulans* HP-2001

Ⅰ.

Medium	g/l	Condition	
K_2HPO_4	5.0	Agitation speed (rpm)	80
NaCl	1.0	Culture time (hr)	60
$MgSO_47H_2O$	0.2	Inoculum size(%)	3
$(NH_4)_2SO_4$	0.6	Temperature (℃)	30
yeast extract	2.5	Inner pressure (kg/cm^2)	0.5
Ascorbic acid	**1**	**Initial pH**	**6.0**
		pH control	**5.0(after 24hr)**
Sucrose	100	Aeration rate (LPM)	1500

Ⅱ.

Medium	g/l	Condition	
K_2HPO_4	5.0	Agitation speed (rpm)	80
NaCl	1.0	Culture time (hr)	60
$MgSO_47H_2O$	0.2	Inoculum size(%)	3
$(NH_4)_2SO_4$	0.6	Temperature (℃)	30
yeast extract	2.5	Inner pressure (kg/cm^2)	0.5
$CaCO_3$	**0.5**	Initial pH	**6.0**
Ascorbic acid	**1**		
Sucrose	100	Aeration rate (LPM)	1500

4. Development of Process for Purification and Separation of Pullulan by *Aureobasidium pullulans* HP-2001

4-1. Development of Process for Purification and Separation of Pullulan Using Filter Press and Active Carbon

4-1-1. Method

First step, the amount of diatomite (Hyancell #200) into R/O water to remove cells by a filter press was 1.25 %(w/v) based on the removal rate of cells. Filter type : Plate & Frame filter press, air permeability : 110 cc/cm^3/min, Dimension of plate : 100 nm x 100 nm x 6 chambers. Second step, The optimal process from supernatant after removing of cells by a filter press was into a active carbon, 0.25 % (w/v). The optimal process for removing cells, melanin-like pigment from culture broth after a filter press were found to be two times of the cycles.

4-1-2. Results

After culture time, the fermentation solution should be through separated and purified recovery process. Because pullulan in the fermentation solution would be degraded easily, so we have to detect the best time of separation and purification. The fermentation solution was stored in 4℃ and 25℃ to detect the pullulan concentration and molecular weight. The result showed that the solution was steady during 24 hr. After 36 hr, the molecular weight decreased a little. And after the culture time, we removed the cells from fermentation solution (Table 8)

The optimal amount of diatomite into culture broth to remove cells by a filter press was 1.25 %(w/v) based on the removal rate of cells (Table 9). The optical density at 520 nm was used to determine the pigments. The optimal process from supernatant after removing of cells by a filter press was 0.26 for optical density. And next step was using active carbon. This method was a simple and efficient procedure for the isolation and purification of pullulan from the cell consisting of active carbon treatment (Fig. 25). Adsorption was effective to separate the cells and melanin-like pigments, 0.023 for optical

Table 8. Variation of culture broth with time elapsed on several times at room temperature. (a) 25℃, (b) 4℃

Temp.	The Time Elapsed(hr)	brix(%)	OD(320)	Mw(x1,000)	Pullulan(g/l)
25℃	0	6.5	1.561	610	41.63
	24	6.4	1.452	623	43.18
	36	6.4	1.575	463	42.24
	48	6.2	1.558	425	44.20
	60	6.4	1.637	355	39.72
4℃	24	6.4	1.362	774	42.14
	36	6.4	1.362	586	40.88
	48	6.2	1.353	603	42.56
	60	6.6	1.402	523	39.70

Table. 9. Separation of *A. pullulans* HP-2001 cells with filter press

Time (min)	Flow rate (l/min)	Pressure (Kgf/cm^2)	OD (320nm)
0	-	0	26.9
2	0.15	4	15.1
4	0.10	5	10.3
6	0.08	6	5.3
8	0.06	6	1.5
10	0.06	6	0.26

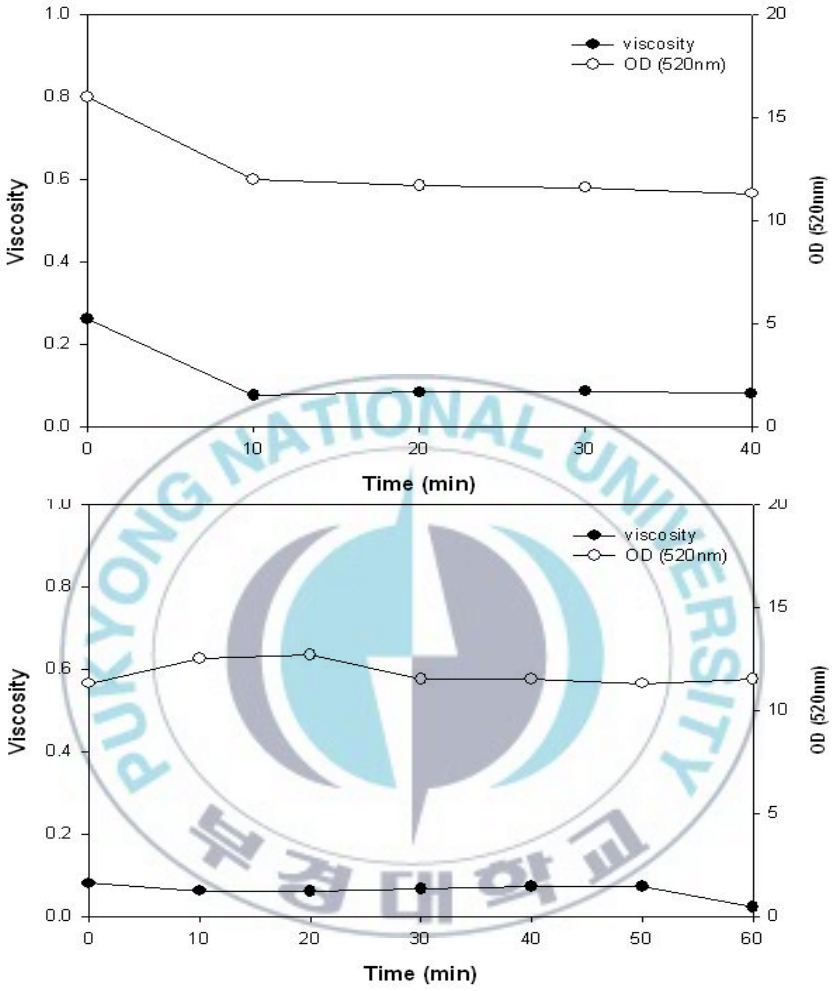
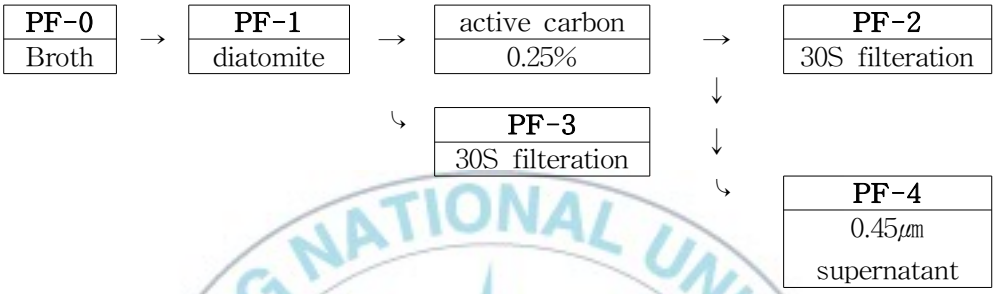


Fig. 25. Separation of *A. pullulans* HP-2001 cells using active carbon with filter press

density of the cells and melanin-like pigments were adsorbed by this method (Table 10).

Table. 10. Development of Process on molecular weight, production for purification and separation of Pullulan by *A. pullulans* HP-2001



	Visco (cps)	brix (%)	OD (520nm)	Mw ($x10^3$)	protein (%)	pullulan (g/l)
PF-0	29.7	7.4	26.92	282	6.03	49.70
PF-1	16	6.9	0.252	119	1.13	35.34
PF-2	11.5	5.4	0.073	146	0.65	30.73
PF-3	6.25	5.0	0.305	119	0.98	30.73
PF-4	6.92	4.8	**0.073**	122	0.32	30.84

4-2. Development of Pullulan purification Process using Ceramic Filtration Membrane

4-2-1. Method

The optimal process from supernatant after removing of cells by a filter press and mixing active carbon by a filter press was to use a ceramic membrane with a molecular cut off size of 50 kDa. (UF ; TAMI Co.) Above 90℃ was chosen for heating temperature for concentration of pullulan.

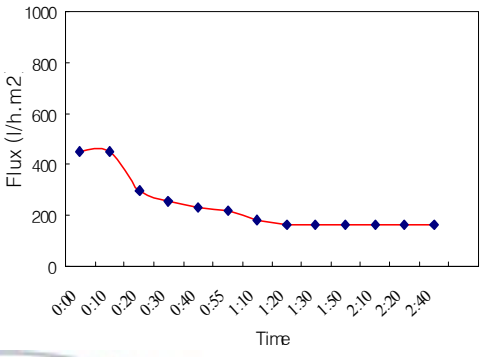
4-2-2. Results

Ceramic filtration membranes are of great interest in separation technology because of their higher chemical, thermal and mechanical stability compared to organic membranes. With ceramic membranes a filtration at high temperature (up to 50 0℃) and extreme pH-value (pH 1-14) is possible. The membranes can be cleaned with aggressive chemicals, organic solvents or hot water steam [82]. UF is gradually adopted substituting filter press for the filtration of the fermentation broths. The application of UF membrane with narrow pore size

not only can remove the solid biomass, but also can substantively reduce emulsion to improve the successive solvent extraction efficiency [83,84]. The optimal process for concentration of pullulan from supernatant after removing of cells by a filter press was to use a ceramic membrane with a molecular cut off size of 50 kDa to remove materials and concentrate pullulan (Table 11).

Table 11. Concentration of pullulan by ceramic ultra-filtration

Time(min)	Temp.	J (l/h.m2)	Sample
0:00	80	450	5L
0:10	81	450	15L
0:20	80	300	25L
0:30	80	255	35L
0:40	80	234	40L
0:55	80	219	50L
1:10	80	180	60L
1:20	81	165	65L
1:30	80	165	70L
1:50	80	165	75L
2:10	80	165	85L
2:20	80	165	90L
2:40	80	165	100L



RETENTATE END VOLUMN	20.0	l
PERMEATE END VOLUMN	100.0	l
TOTAL VOLUMN	120.0	l
AVERAGE FLUX	192.3	l / h.㎡

4-3. Development of Process of Freeze Dryer and Structural Characterization of Pullulan

4-3-1. Method

Freeze-drying requires the use of a special machine called a freeze-dryer, which has a large chamber for freezing and a vacuum pump for removing moisture. The product is frozen. This provides a necessary condition for low temperature(-70℃) drying. plate temperature was 20℃ for 48 hr.

Pullulan sample (powder) was determined FTIR. FTIR spectra were recorded with a Perkin-Elmer 1720 spectrometer over KBr pellets. Pullulan sample (2 mg) was well blended manually with 100 mg of KBr powder. This mixture was then desiccated overnight at 50℃ under reduced pressure prior to FTIP. Pullulan-J made in Hayashibara CORP. Pullulan-K made in our sample.

4-3-2. Results

The product is frozen. This provides a necessary condition for low temperature(-70℃) drying. Plate temperature was 20℃

for 48 hr. Its drying rate was 95 %.

Among all these treatment, pullulan concentration and molecular weigh was checked The result showed that pullulan concentration and molecular weight decreased dramatically after the removing of cells by filter press, because the loss was the membrane combination of acidic polysaccharide (Fig. 26). And The FTIR spectra of pullulans produced in Hayashibara CORP. (pullulan-J) and in made ours lab (pullulan-K) exhibited similar features (Fig.27, 28). The strong absorption at 3,380 cm^{-1} indicated that all the pullulans had some repeating units of -OH as in sugars. The other strong absorption at 2,300 cm^{-1} indicated that a sp^3 C-H bond of alkane compounds existed in all the samples.

Based on instrumental and enzymatic analyses, the pullulan produced by *A. pullulans* HP-2001 seemed to have the same basic structures, but the ratios of their monomeric components were a little different, which might have been the reason for the production of pullulans with different molecular weights.

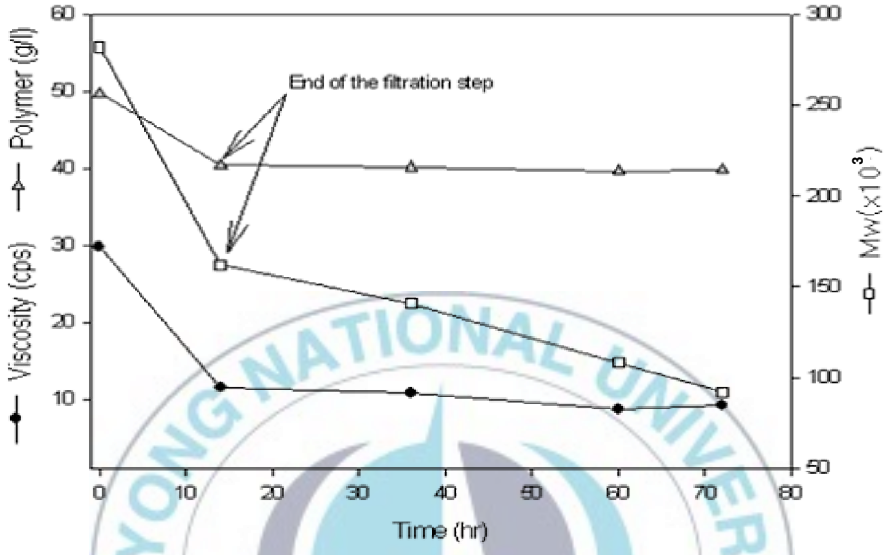


Fig. 26. Process for recovery of pullulan produced by *A. pullulans* HP-2001 with time elapsed

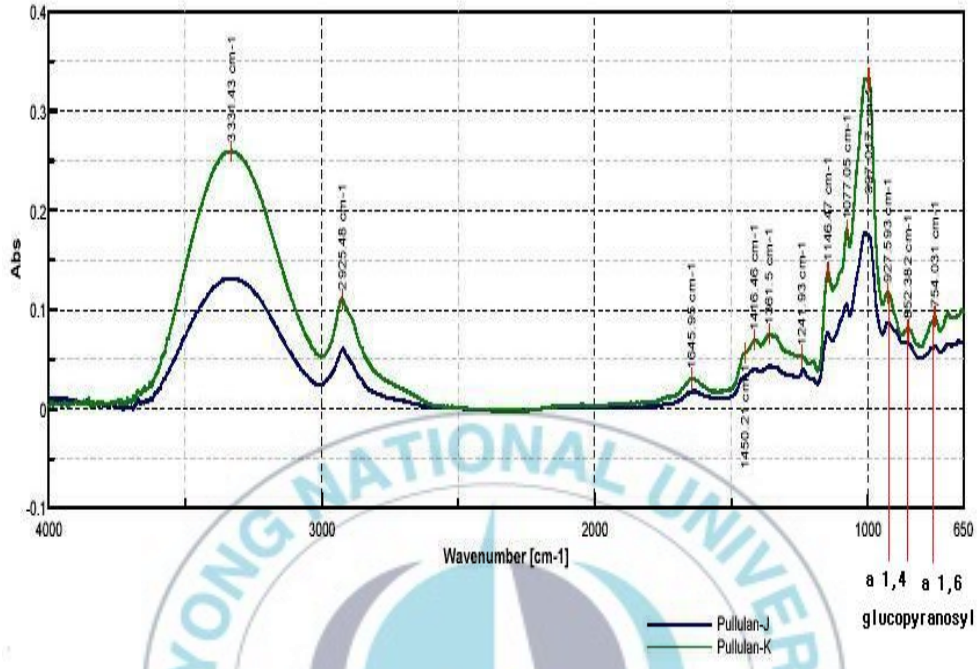
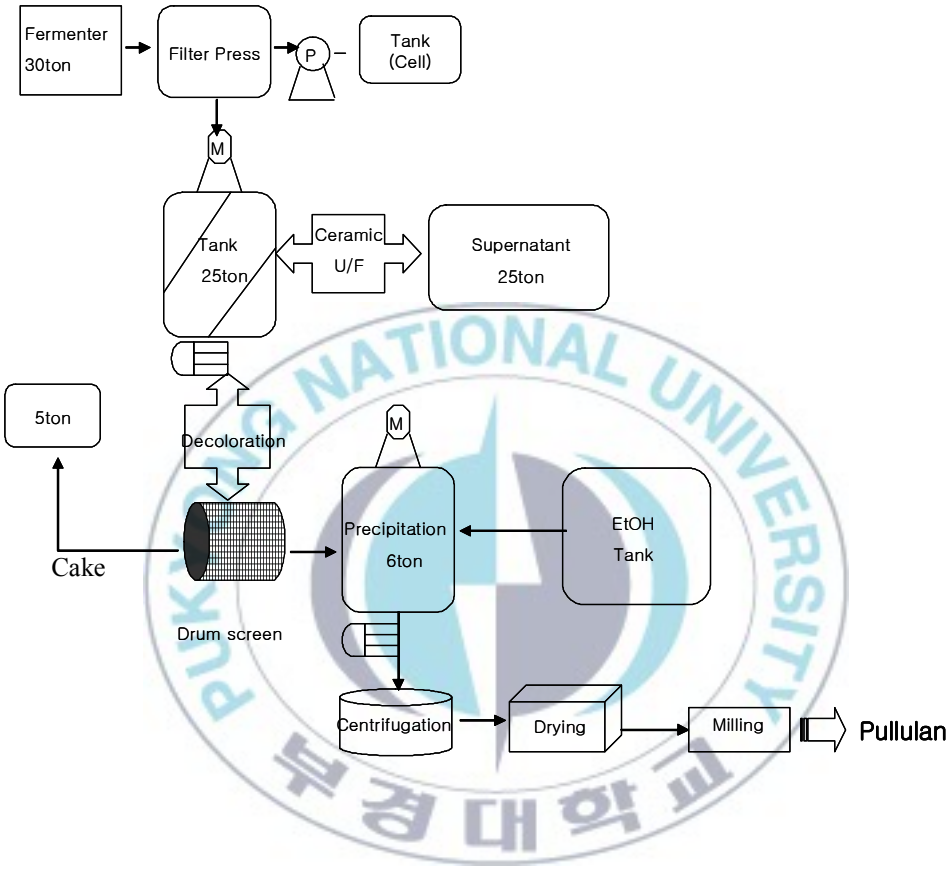


Fig. 27. FTIR spectra of pullulan produced by *A. pullulans* HP-2001

Fig. 28. Flow diagram for downstream processing of pullulan



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뒤늦게 박사를 진학하면서 용기와 격려를 아끼지 않았던 서형필박사, 이남규박사, 진혁박사 그리고 형님같은 황희선 사장님의 깊은 우정에 감사드리고 우리회사의 대표이자 파트너인 손창우 박사에겐 그동안 진학을 위해 배려해준 모든 일들에 감사의 말씀을 드리고 싶습니다. 또한 부족한 선배를 도와주느라 고생해준 이유정 박사, 보경, 정무, 실험실 채훈, 미란, 승준, 지숙, 석주에게도 감사의 말을 전하고 싶습니다. 그밖에 열심히 산업현장에서 혹은 연구에 임하고 있는 저를 아는 선후배님들에게도 감사의 마음을 전합니다. 항상 바쁘다는 핑계로 자주 보지 못했던 친구들과 동아리 선후배님들에게도 미안하다는 말과 함께 감사의 뜻을 전하

고 싶습니다.

그동안의 학교생활을 지켜보시면서 속으로 애타하시며 항상 걱정하셨고, 언제나 저를 믿어주시던 아버님, 어머님, 그리고 형제들께 지면을 통해서 나마 평소에 하지 못했던 감사의 마음을 전하고 싶습니다. 사위를 친자식처럼 걱정해주시던 장모님께도 마음속 깊이 감사드립니다. 마지막으로 언제나 정성어린 격려와 보살핌, 인내로써 지켜봐준 아내 영아와 사랑하는 아들 재민과도 이 기쁨을 나누고 싶습니다.

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